PhD Thesis

PROTEOMIC TOOLS IN CLINICAL TISSUES: UNLOCKING THE PATHOLOGY ARCHIVES

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<tbody>
<tr>
<td>2D-PAGE</td>
<td>Two-Dimensional Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>AT</td>
<td>Archive Tissue</td>
</tr>
<tr>
<td>BMs</td>
<td>Biomarkers</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>BS</td>
<td>Bloom’s Syndrome</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>BTA</td>
<td>Bladder Tumour Antigen</td>
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<tr>
<td>CIN</td>
<td>Cervical Intraepithelial Lesion</td>
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<tr>
<td>CIS</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3-Diaminobenzidine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Serum Albumin</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin Fixed and Paraffin Embedded</td>
</tr>
<tr>
<td>FPE</td>
<td>Fixed and Paraffine Embedded</td>
</tr>
<tr>
<td>FPPA</td>
<td>Forward Phase Protein Array</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HPF</td>
<td>High Power Magnification</td>
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<tr>
<td>HPV</td>
<td>Human Papilloma Virus</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous Recombination</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HSIL</td>
<td>High grade Squamous Intraepithelial Lesion</td>
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<tr>
<td>hTERT</td>
<td>Human Telomerase Reverse Transcriptase</td>
</tr>
<tr>
<td>HU</td>
<td>Hydroxiurea</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>ICGEB</td>
<td>International Center for Genetic Engineering and Biotechnology</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IP</td>
<td>Proliferation Index</td>
</tr>
<tr>
<td>ISH</td>
<td>In Situ Hybridization</td>
</tr>
<tr>
<td>LSIL</td>
<td>Low grade Squamous Intraepithelial Lesion</td>
</tr>
<tr>
<td>MoAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>MP</td>
<td>Milk Powder</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
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<tr>
<td>O.M.</td>
<td>Optical Magnification</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Pleating Efficiency</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RPPA</td>
<td>Reverse Phase Protein Array</td>
</tr>
<tr>
<td>RTS</td>
<td>Rothmund-Thomson Syndrome</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>SEB</td>
<td>Surrogate Endpoint Biomarkers</td>
</tr>
<tr>
<td>SIL</td>
<td>Squamous Intraepithelial Lesion</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue Microarray</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolamide</td>
</tr>
<tr>
<td>TRAP</td>
<td>Telomeric Repeat Amplification Protocol</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>WS</td>
<td>Werner’s Syndrome</td>
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ABSTRACT

Background

Clinical proteomics aims to characterize the protein networks altered by pathologic processes or therapeutic treatment, and to develop biomarker profiling technologies to promptly detect diseases and treat them more effectively. The challenge of translating proteomic profiling to the bedside lies in applying technologies for the analysis of human tissues, which are routinely obtained by biopsy or surgery, without substantially modifying the clinical workflow. Formalin-fixed and paraffin-embedded (FFPE) tissues are the most widely available archive material suitable to discover new biomarkers or therapy targets, for their validation and for their implementation in clinical practice. However, the lack of standardised guidelines for protein analysis of archive tissues has hampered their regular use in the daily practice. Furthermore, methods to extract proteins and to identify and analyse them also quantitatively have only recently been developed.

This research was carried out to develop, standardise and apply qualitative and quantitative proteomic methodologies to archive tissues.

Objectives

1. Development, standardisation and validation of protein extraction methods and protocols from fixed and paraffin-embedded tissues with different pre-analytical conditions.

2. Application of protein analysis in two different research settings:
   a. at diagnostic level: screening of monoclonal antibodies against Human papillomaviruses (HPVs) in cervix uteri lesions suitable for early detection and progression risk assessment;
Abstract

b. at translational research level: identification of a new therapy target for glioblastoma, deepening the functions of human RECQ1 helicase, an enzyme involved in the maintenance of chromosome stability.

Methods

1. In order to develop, standardise and validate protein extraction methods and protocols from fixed and paraffin-embedded tissues, lung, colon and breast cancer specimens were collected by three groups from the European consortium IMPACTS (Integration of Molecular Pathology and Cell and Tissue Structure). Each specimen was divided into two equivalent sets: one was fixed in formalin and paraffin-embedded; the other one was fixed in a new alcohol-based fixative (FineFix) and paraffin-embedded. In order to compare the fixation procedures and the protein extraction efficiency, a total of 81 protein lysates were prepared in five different laboratories of the consortium. Selected marker proteins were compared, at quantitative level, between the two fixation procedures using the reverse phase protein array technology. Tests were applied to determine whether any of the fixation methods caused differences in protein lysate microarray measurements. Pairwise differences between fixation methods were tested.

2. (a) In diagnostics, protein analysis was applied to 138 FFPE cervix uteri squamous lesions provided by a multicentric retrospective study. They were characterized at molecular level for the presence and type of HPV by using PCR-based systems or the Hybrid Capture assay. Thirty-nine monoclonal antibodies directed against the E7 viral oncoprotein were supplied by a private company. They were validated by generating different tissue microarrays in which multiple punches for each bioptic sample were done, taking different cervical lesions (from low grade to invasive squamous cancer). The antibody providing better staining and specificity results was selected and validated with other tissue microarrays, which included a total of 138 tissue cores from different cervical
Abstract

lesions, and with different HPV-types infections. HPV capsid protein L1, the surrogate biomarkers p16, hTERT, p53 and Ubiquitin, and the proliferative marker Ki67 were also tested in the same way. For the selected E7 antibody and the six biomarkers, one-way analysis of variance was performed to detect significant differences in the mean number of positive cells and in the mean staining intensity (evaluated by a score from 0 to 3+). The analysis was carried out among different types of cervical lesions, both at cytoplasmatic and nuclear level, by epithelial layer. We made use of repeated-measure analysis of variance to ascertain significant differences in the mean number of positive cells between E7 monoclonal antibody and each biomarker. Spearman’s rank correlation test and linear regression analysis were employed in order to detect correlations between E7 and the other biomarkers.

2. (b) The application of proteomics to FFPE tissues at translational research level concerned the expression of the RECQ1 helicase in glioblastoma and in perilesional brain tissue, and its expression in lung, colon and thyroid carcinomas and in their perilesional tissues. All specimens were submitted to immunohistochemistry. The expression pattern of RECQ1 was further analysed on a tissue microarray containing 63 glioblastomas and 19 perilesional brain tissues. Furthermore, to test the function of RECQ1 in glioblastoma growth, RNA interference experiments were performed in different glioblastoma cell lines and the effect of gene silencing on cell growth and proliferation was monitored. The Wilcoxon test for matched-samples was performed to evaluate differences in immunohistochemistry outcome measures, i.e. the proportion of positive cells for RECQ1, the intensity of the signal and the number of positive cells for Ki67, between tumour and perilesional tissues. Spearman’s rank correlation test was applied to assess the relationship between intensity score of RECQ1 and the number of positive cells in tumour specimens. Kruskal-Wallis test was carried out to investigate differences in the proportion of positive
cells for RECQ1 and Ki67 among groups defined by patient gender and by RECQ1 intensity score.

Results

1. Quantitative comparison between fixatives for the tested antibody panel (β-Actin, E-cadherin, EGFR, HER2 and ER) yielded a higher immunostaining intensity for the FineFix lysates compared to the FFPE ones (p = 0.04). Signal intensities for EGFR (p = 0.007) and HER2 (p = 0.02) were significantly higher in the FineFix samples compared to the FFPE samples, whereas no differences were observed for β-Actin, E-cadherin and ER.

2. (a) The E7 immunostaining was similar to that of p16, which is the most widely-used surrogate marker in clinical practice for all types of cervical squamous lesions. Furthermore, in high grade squamous intraepithelial lesions (HSILs) E7 was similar to hTERT. In the detection of squamous cell carcinoma, E7 was similar to hTERT and Ubiquitin. A comparison between the mean number of E7 and L1 positive cells showed a significant difference in low grade squamous intraepithelial lesions (p = 0.002), among HSILs (p = 0.01) and in squamous cell carcinoma (p = 0.01). Correlation analysis between the two viral proteins, E7 and L1, allows to identify two possible groups of lesions, both in the low grade squamous intraepithelial lesions (LSILs) and in the HSILs. One group was characterized by high level of E7 and low level of L1, the other group was characterized by high level of both marker. This groups were also re-evalueted at morphological level and lead us to hypothesize two possible models in the progression of LSILs: the viral replicative model and the cell proliferative one.

2. (b) RECQ1 was highly expressed both in the perilesional sections and in lesional sections of all tumours analyzed, with the exception of glioblastoma where its expression was significantly higher in tumour (p = 0.001). RECQ1 expression was confined in the nuclei of the tumour cells, thus suggesting that this enzyme might play an important role in
glioblastoma growth. As expected, immunohistochemical analyses against Ki67 showed that protein expression was higher in glioblastoma than in the surrounding normal tissue ($p = 0.0008$). No correlation between the proportion of positive cells for RECQ1 and Ki67 was found (Spearman’s rho = 0.2, $p = 0.2$), suggesting that the high expression of RECQ1 in brain tumours is not simply related to the higher degree of proliferation of these cells. An essential role of RECQ1 in tumour growth and proliferation was confirmed by RNA interference experiments and clonogenic assay on cell lines.

**Conclusions**

During my doctorate, all the objectives set were obtained. The goals achieved through this research are the following:

- The standardization of a suitable protein extraction protocol from FFPE tissues. This study demonstrated that it is possible to harmonize protein analysis in archive tissues in different European laboratories by using the same protocols for tissue processing and protein extraction.

- The optimization of an appropriate protocol for molecular analysis of tissues fixed with new formalin-free fixative FineFix. The possibility of using proteomic approaches also on tissues fixed with reagents alternative to formalin has great potential for future pathology, when the use of formalin will be banned due to its carcinogenic effects. Furthermore, FineFix has proven a suitable formalin substitute in clinical practice, because it preserves both specimens’ morphology and immunoreactivity.

- The validation of a new antibody against the E7 oncoviral protein for the detection of HPV in cervical squamous lesions. This leads us to conclude that E7 might be a suitable specific marker for the diagnosis of cervical lesions and could be used also for others HPV-linked
diseases. The analysis of a battery of E7 monoclonal antibodies has highlighted their great usefulness in differential diagnosis.

- An in-depth knowledge of the function of RECQ1 in glioblastoma. RECQ1 expression has been studied in glioblastoma cell lines for the first time ever. This research has demonstrated its important role in glioblastoma growth and proliferation, and in maintaining genome stability.

- The recommendation of RECQ1 helicase as therapy target in glioblastoma, based on the evidence that the enzyme expression in this tumour type is higher than in perilesional normal tissues or in cancers of different origins. These findings lead us to consider RECQ1 a reliable target for the development of new anti-cancer therapies to eliminate proliferating tumour cells.

Finally, this research demonstrated that archive tissues could be a valuable source of material for proteomics research.
INTRODUCTION

Traditionally in medical practice, a disease is characterized on the basis of the symptoms that patients show at the level of the whole body and/or at organ level. With the development of new technologies, there is a growing trend in using molecular analyses to better define diseases. The translation of new knowledge into tangible clinical benefit is essential to raise standards of healthcare and to develop diagnostic, prognostic and new therapeutic intervention strategies for the implementation of tailored medicine and the improvement of patient management (1). The translation of molecular techniques in clinical application is also one of the most challenging goal of the major world organizations working in this field, such as the Food and Drug Administration (FDA, http://www.fda.gov) and the National Institutes of Health (NIH, http://www.nih.gov) in the United States, and the Innovative Medicine Initiative (IMI, http://imi.europa.eu) in Europe.

1. MOLECULAR MEDICINE: TOWARDS A MOLECULAR PATHOLOGY APPROACH

Molecular medicine” is commonly referred to as a rapidly growing multidisciplinary approach aimed at understanding the biological features of diseases, in order to translate evidence from molecular biology into clinical practice. It involves many different disciplines and expertise in various biological fields, such as biochemistry, cell and molecular biology, genetics, functional genomics, proteomics and immunology. The development of biotechnologies has facilitated the comprehensive analyses of genomes, transcriptomes and proteomes in healthy and ill subjects. Innovations and applications of biotechnology have developed tools devoted to manage disease treatment as well as to support diagnosis, prognosis and prevention. In particular, molecular medicine proves useful in identifying disease subtypes which are not recognizable by classic pathologic criteria and in identifying specific genetic events involved in the pathogenesis of diseases (2).

Oncology is one of the fields that has shown more interest in translational research. Cancer is one of the leading causes of mortality in western society and huge efforts have been made to fight this
condition. In Europe, 3.2 million new cases of cancer and 1.7 million deaths from cancer were estimated in 2008 (3).

Worldwide, histological diagnosis of cancer is currently performed on formalin fixed and paraffin embedded (FFPE) tissues, using approaches based on a combination of histopathology and immunohistochemistry. Currently, the most widely and accurate systems for staging cancer are the TNM and pTNM classifications (T for Tumour, N for Node, M for metastases, p for pathologic), which are defined by macroscopic and microscopic examinations of the tumour and histological evaluation of its slides (http://www.cancerstaging.org) (4-9). These systems provide precise information on primary site, tumour type, stage and grade as well as information on the completeness of surgical tumour removal. Up to now, these data include the most relevant information on a patient’s prognosis and are a rational basis for therapy design (10). However, cancer is a heterogeneous disease, and tumours characterized by the same histological type may have different outcomes. This means that classic pathological criteria are not always sufficient to properly define these lesions. Thus, new tools are needed for sub-classify cancer lesions. Thanks to progress in high-throughput “-omics” technologies, human bio-specimens can be successfully used for wide analysis at all molecular levels (DNA, RNA and proteins), and the identification of novel tumour sub-classes. Recently, molecular classification has been used to identify unique sub-classes of cancers, including acute myeloid leukemia (11, 12), glioblastoma (13, 14), breast cancer (15, 16) and renal cell carcinoma (17, 18), and to differentiate between Burkitt’s lymphoma and diffuse B-cell lymphoma (19).

Molecular analyses can also be very effective in correctly addressing the use of very expensive drugs, so that they can be administered only to those patients that can really benefit from them. For instance, gene-expression profile analyses has identified four major breast cancer phenotypes: luminal A, luminal B, Her2-like, and basal-like. Both luminal A and luminal B subtypes are generally estrogen receptor positive and, therefore, candidates for treatment with drugs such as tamoxifen. The luminal B subtype has an increased expression of genes associated with cell proliferation.
Gene expression profile can also provide information on the prediction of clinical outcome, *e.g.*, Luminal B tumour have a poorer overall outcome than the luminal A subtype (20, 21). Several tests have been developed to support clinical decision (Figure 1) (22). For example, in breast cancer, the mammaPrint® test (23) measures the expression of 70 genes based on a set of 78 patients with node-negative breast cancer who had received no adjuvant therapy. It provides a prognostic score that stratifies patients into good or poor prognosis groups, and can be used on fresh or frozen tissue. Another example is the oncotype DX® test (24) that measures the expression of 21 genes by real-time PCR and can be used on formalin-fixed paraffin-embedded tissue. This test predicts the risk of recurrent disease in ER-positive, node-negative breast cancer patients and categorizes patients into low, intermediate or high risk of recurrence. Low risk patients would not receive chemotherapy whereas high-risk patients would.

When these technologies are combined with microdissection procedures, it is possible to perform selective analysis of DNA, mRNA or proteins from precise locations within a tumour and to achieve the definition of the malignant cells’ specific molecular pattern. For example, microdissection enhanced expression profiling of breast (25-27), ovarian (28-31) and prostate cancer (32-36).

These innovative molecular techniques do not supplant the traditional approaches, but they represent valuable tools for complementing the current diagnostic and prognostic approaches. The ability to sub-classify cancers into molecular groups and treat them more effectively will significantly improve patients management.
2. CLINICAL SAMPLES IN MOLECULAR MEDICINE

In the last years, an increasing number of reports on the clinical application of genomics and proteomics research for the identification of biomarkers and therapies targets have been published (10, 28, 37-39). Human specimens are more appropriate than cell culture systems to develop new diagnostic, prognostic and predictive biomarkers. For example, cell models available from the American Type Culture Collection (ATCC) repository are powerful tools that have led to the discovery of many cellular processes. However, some studies questioned the validity of cultured cells as accurate global expression models of human cells in vivo. For example, Celis and co-workers found that short-term culturing of bladder cancer cells led to changes in expression of
several proteins involved in key cellular activities (40). Another group used two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis in order to compare proteomic profiles of two widely used prostate epithelial cell lines with prostate epithelial cells directly microdissected from a human prostate gland. They found that tissue samples were significantly dissimilar from the cultured lines, even at the level of high abundance proteins (41). These examples indicate that cell lines might not fully reflect human pathologic tissues due to the lack of the tissue (micro)environment. As a consequence, the use of clinical specimens is always necessary to characterize molecular alterations involved in human diseases (42).

Human clinical samples used for translational research may be obtained from healthy or ill subjects, alive or dead. Some human material is donated by volunteers also for research (e.g. blood sample), but tissues removed during surgical treatment and surgical left-overs can also be useful sources. There are two main categories of human material that can be used in molecular medicine research: biological fluids e.g., serum, plasma, urine, cerebrospinal fluid, and tissue specimens. The collection of blood, urine and stool is usually unproblematic and can be carry out repeatedly, even in the same patient. Conversely, collecting tissues specimens is always invasive and is always restricted to diagnostic and therapeutic procedures. FFPE samples can be used for histological analyses and molecular studies on DNA, RNA and proteins. Fresh-frozen tissues are ideal for biomarker profiling and prospective molecular analyses, because macromolecules are intact. However, they can lead to less accurate microscopic diagnosis and are difficult to handle as a routine material in most pathology department. Furthermore, this kind of tissues is not suitable for retrospective studies because of inadequate follow-up period.

On the other hand, FPPE tissues are valuable resources for retrospective diagnostic and research studies. In the clinical practice, formalin fixation and paraffin embedding is the standard procedure for the preparation of samples for histopathological assessment. The tissue specimens are fixed as soon as possible to avoid autolysis and putrefaction and then they are paraffin-embedded to allow the cutting of very thin sections for histological examination. Subsequently, some sections are cut
from the paraffin block and these are stored in the pathology archive for decades (for this reason they are called archive tissues, AT). This material can also be matched with patient’s clinical records. It is an affordable and highly characterized type of human tissues biobank where even rare diseases are available for translational research. Moreover, AT represents actual pathological material and is very useful for clinical and population-based molecular epidemiological studies. Thus, FFPE tissues are the most widely available material for clinical retrospective studies. They can also be used in the discovery phase of new biomarkers or therapy targets, for their validation and for their implementation in clinical practice. Normal tissues surrounding the lesion or from biopsies without pathological findings are also available. AT specimens have some limitations in comparison to fresh frozen samples, because of the chemical modifications performed by formaldehyde on nucleic acids and proteins (43). However, compared to fresh frozen tissues, AT have three main advantages: (1) low cost, since they are still stored in the pathology department; (2) tissues are morphologically well-defined and referred to a specific clinical diagnosis performed by experienced pathologists; (3) the huge number of samples available with even rare lesions and long follow-up. Hence, the use of AT in clinical research can accelerate the translation of the molecular biology knowledge to molecular medicine.

2.1. APPROACHES AND TOOLS IN ARCHIVE TISSUES

In the past, the main molecular application of AT was immunohistochemistry and in situ hybridization-based techniques. However, these methods are semi-quantitative and allow only a few targets to be evaluated at a time. Recently, the possibility of applying new molecular techniques to DNA, RNA and protein also from FFPE tissues offers a unique opportunity to detect and quantify biomarkers, and to improve diagnosis, prognosis and therapeutic decisions. However, molecular analyses have some limitations, due to the extensive degradation of macromolecules before and during formalin fixation (44). Formalin fixation results in the formation of chemical cross links between RNA/DNA and protein, and in the addition of monomethylol groups to nucleotide base pairs. This process increases the susceptibility of nucleic acids to shearing and
fragmentation. Also protein analyses are impaired by formalin which can modify them by producing protein-protein cross links and reducing the amount of extractable material (45). Moreover, the lack of standardized guidelines for the DNA, RNA and protein analysis of AT seems to have hampered the use of such samples for molecular analyses. In 2007, pathologists from 20 different European centres participated in the European project “Archive tissues: improving molecular medicine research and clinical practice” (IMPACTS¹). IMPACTS gave a consistent solution to the difficulties and complexities in translating molecular pathology research into clinical practice through the use of archived tissues. Joint efforts also produced a book of guidelines to be published in 2011 for a reliable clinical application of molecular approaches in archival material (46).

3. CLINICAL APPLICATION OF MOLECULAR MEDICINE

The application of molecular methods in clinical practice and in personalized medicine has produced the identification and validation of biomarkers. In 2001, the National Institutes of Health's Biomarkers Definitions Working Group defined a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (47). Thus, biomarkers are important molecular signs of cell phenotype and could be very relevant in diagnostics and in the decision of clinical interventions.

¹The European project IMPACTS (contract no. LSHG-CT-2007-037211) was coordinated by our laboratory in Trieste. For more details: http://www.impactsnetwork.eu,
3.1. CLASSIFICATION OF CANCER BIOMARKERS

Genomic and proteomic technologies have significantly increased the number of potential DNA, RNA and protein biomarkers under investigation, allowing their detection both in human fresh frozen/archival tissues and in biological liquids. The identification of biomarkers is important in modern medicine, especially in oncology. Biomarkers are classified as follows.

*Risk biomarkers* reflect increased cancer risk by type or subtype of neoplasm. They could reveal also the existence of premalignant lesions and early-stage cancers. For example, individuals with inherited mutations in the DNA mismatch repair genes MLH1 and MSH2 have a higher risk to develop colon cancer (48). Knowledge of their predispositions can promote screening colonoscopy with the aim of early cancer detection and treatment.

*Predictive biomarkers* (or *response markers*) are utilized to assess the effect of administering a specific drug. For example, only those lung cancer patients harbouring specific mutation in the EGFR gene (either exon 21 point mutations or exon 19 deletions) are responsive to EGFR inhibitors, such as gefitinib and erlotinib (49). These response markers can also predict treatment dose and safety in different sub-groups of cancer patients. Variations in genes that encode drug-metabolizing enzymes, drug transporters or drug targets can have detrimental effects on patient treatment outcomes (50). In familial breast cancer, genetic variations in CYP2D6, the cytochrome P450 enzyme, have been shown to reduce survival in individuals treated with the chemotherapeutic drug tamoxifen (51).

*Diagnostic biomarkers* are used to evaluate individuals with evidence of cancer, and they can be measured at any stage of cancer development. For example, a rising level of prostate-specific antigen (PSA) is indicative of prostate cancer. For bladder cancer, the FDA had recently approved some diagnostic marker to be used in urine analyses, such as bladder tumour antigen (BTA) and
nuclear matrix protein-22 (NMP-22) (52, 53). Survivin and calreticulin also have diagnostic potential in this type of cancer (54, 55).

**Prognostic biomarkers** predict outcomes in patients with a specific disease. Such markers can be useful in stratifying patients into distinct prognostic risk groups and to guide therapy decision. By using tissue microarray analyses, Kim et al (56) constructed a combined molecular-clinical prognostic model to predict survival probability for patients with renal cell carcinoma. This model was significantly more accurate than standard clinical parameters. The above mentioned Oncotype Dx Breast Cancer Assay has also proven to be a good risk predictor of breast cancer recurrence in young women with negative lymphnodes (57).

When a biomarker is a good predictor of clinical risk or benefit, it may achieve the status of surrogate (“to substitute for”) endpoint (**Surrogate endpoint biomarkers**, SEBs). In these circumstances, this biomarker is able to substitute the corresponding clinical endpoint that the conventional diagnostics normally uses in the assessment of a specific therapeutic intervention (47). For example in cervical cancer, since Human Papilloma Virus (HPV) is critically important in the carcinogenesis process, several SEBs have been studied in relation to HPV infection and to its life cycle. Many of these are related to cell proliferation or to cell cycle control such as Ki67 and p16 (58). Although all surrogate endpoints can be considered biomarkers, only a few biomarkers will achieve surrogate endpoint status.

The clinical use of biomarkers is connected to the notion of their sensitivity, specificity and predictive value. An emerging issue in biomarker research is the multiplicity, referred to the use of panels of biomarkers, rather than a single one. This seems to increase both sensitivity and specificity especially in the pre-symptomatic detection of cancer (59). In this context, the prevalence of early stage disease and the costs of false results (both positive and negative) must be weighed against the benefits of early diagnosis. An ideal screening test would have very high
sensitivity, identifying nearly all individuals with the disease. It also might have a high specificity to avoid unnecessary misclassification and expensive medical follow-up (38).

The use of biomarkers in clinical practice can be fully achieved only through an intensive collaboration between laboratory researchers and clinicians as it requires a continuous interchange of clinical and molecular information. This cooperation is essential in all steps of biomarker analysis, from laboratory detection to the final validation, in order to develop less expensive and more effective screening tests.

3.2. CLINICAL PROTEOMICS

Among the molecular “-omics” technologies, proteomics plays a central role in molecular medicine offering great promise for molecular diagnostic medicine. Cancer, although often classified as genetic disease is, functionally, a proteomic disease. Genetic mutations can indeed modify signalling pathway. They can create a survival advantage for the cells forcing to ignore negative inhibitory signals, or can perpetually send them false positive signals. Therefore, genetic abnormalities in tumour cells, which are measured through DNA and RNA analyses, do not portray the situation at the protein level. Understanding the functional changes in the human proteome, which arise from the genomic alterations, is the next logical step in the development of high-value biomarkers that can be transitioned to clinical studies (39, 60-66). The ultimate goal of clinical proteomics is really twofold: (a) characterizing the protein networks altered as a consequence of pathologic processes or after therapeutic treatment; (b) developing biomarker profiling technologies to detect a disease earlier and treat it more effectively (67). In contrast to the genome, the cellular proteome is dynamic, including differential splicing of the relative mRNAs, post-translational modifications and temporal and functional regulation of gene expression. For these reasons, although studies of differential mRNA expression are informative, the concordance among mRNA expression profile and protein function is not linear. In literature different studies have emphasized this evidence (68-80).
In this scenario, we can foresee the use of proteomic analyses at many steps of the natural history of diseases as follows:

- improved classification of cancer, complementing the histopathology;
- a better understanding of disease molecular pathology, particularly in cancer;
- improving the molecular diagnosis and the prognosis of cancer and other diseases;
- facilitating the integration of diagnostic and therapeutic toward personalised therapy.

Currently, the development of techniques for the analysis of proteins in clinical tissues is tricky. The human proteome is estimated to be at least 50 times the number of human genes, due to the post-translational modifications which can occur in different combinations and on different splice variants (81). Proteins of clinical interest could vary highly in concentration levels depending on the type of specimen and on the protein function. Amplification methods like polymerase chain reaction (PCR) for nucleic acids have not been developed for proteins. DNA has a rather simple structure in comparison to proteins. Proteins, which are composed by the combination of 20 amino acids, can be hydrophobic or hydrophilic, acid or basic. The specificity and sensitivity of a protein analytical system are further critical points of interest. Highly specific and selective capture molecules, e.g., antibodies, are absolutely needed, but are rarely available for clinical use. Moreover, precise quantification of protein expression for every tumour is also required for clinical purposes. Numerous proteomic technologies are available - most of them are mass spectrometry based (e.g., SELDI-TOF) - but only few of them are relevant to clinical practice and to AT application.

Antibody-based proteomics could provide the strategy to translate such approaches to molecular diagnostics (82-84). Two examples of these applications are the Human Protein Atlas (HPA, [http://www.proteinatlas.org](http://www.proteinatlas.org)) and the Clinical Proteomic Technologies for Cancer (CPTAC, [http://proteomics.cancer.gov](http://proteomics.cancer.gov)) (85-87).
PROTEOMICS METHODS IN ARCHIVE TISSUES

Proteomic analysis of tissues will have a central role in the identification of specific biomarkers for diagnosis, prognosis and in assessing disease progression risk. Moreover, since proteins are drug targets themselves, they can be useful even in the choice of therapy. Quantitative protein analyses in AT is necessary in the clinical practice since a precise measurement of a specific protein expression level could help clinicians to better define a more appropriate molecular-targeted therapy. For example, the receptor tyrosine kinase HER2 is a therapeutic target for breast cancers overexpressing HER2. Thus, the quantitative determination of HER2 protein in breast cancer tissues could help the therapeutic decision. In the past, FFPE tissues have been assumed to be unsuitable for proteomics studies but immunohistochemistry. Immunohistochemistry (IHC) has been the method of choice to investigate and test biomarkers in FFPE tissues. As a consequence, at present IHC represents the only tool to define a proper diagnosis and treatment at protein level. For many years IHC has been semi-quantitative, the main limitation of this technique. Recently, IHC has become quantitative through the development of innovative heat-induced antigen retrieval methods, which have been successfully utilized as protein extraction method from FFPE tissues for Western blot (88-94), mass spectrometry (90, 95-97), and reverse phase protein lysate microarrays (91, 94, 98, 99). The great advantage of these extractive methods consists in the possibility of analysing FFPE tissues quantitatively. Hence, the integration of proteomics methods in routine practice would be possible, without modifying the clinical workflow. A successful extraction protocol in routine diagnostic should be fast, effective, standardized, and reliable. However, this material is submitted to a series of essential treatments which can directly affect their use in downstream molecular studies. All these procedures are named pre-analytical treatment and include tissue fixation, preservation and sample collection. In particular, the fixation step is of crucial importance because the quality of macromolecules that we can obtain from FFPE tissues varies according to the time of fixation which is not fully standardized (for example, there is difference between samples collected during the week and just before the weekend or during
holidays). This step is particularly important because the number of cross-links among proteins increases over time. Extendedly fixed tissues represent a hurdle for efficient protein extraction.

During my doctorate, I addressed some of these issues, developing and applying some proteomics technologies to clinical samples, especially AT.

In the following sections I will describe the most commonly used proteomic methods and platforms in AT.

### 3.3.1. TISSUE MICROARRAYS

The tissue microarray (TMA) procedure was developed by Kononen et al. (100) as a high-throughput tool to investigate a variety of biomarkers in multiple tissue specimens simultaneously. To construct a TMA small cores of tissues (0.6 -2.0 mm), punched from a donor paraffin block, are transferred into a recipient block in arrayed fashion. Using these cores, samples from hundreds of different tissues or patients can be arrayed in a new paraffin block. The resulting TMA block is then sectioned and analyzed by IHC, *in situ* hybridization (ISH) or immunofluorescence (IF). As research tool, TMAs are predominantly used for the investigation of putative prognostic and predictive molecular targets in human cancer tissues (101). They are used also for *in situ* validation of candidate markers identified in genomics and proteomics studies (102-105) and to correlate staining results with clinical endpoint (106, 107). Moreover, it is possible to perform the so-called progression TMAs, in which cores of a single tissue type are used taking into account different stages of tumour development or different tumour grades. For example a progression TMA for cervix uteri would include normal cervical epithelium, epithelium with low and high grade of dysplasia, as well as invasive carcinomas. TMAs can be used also in experiments aimed to determine whether a protein is expressed or not and its extent in a wide range of different normal and/or pathological tissues. TMAs are also being used for testing new antibodies or determining optimal staining conditions, collecting biopsies from samples representative of diverse or specific lesions. In clinical research, TMA is an important tool because it eliminates slide-to-slide variation,
reduces the amount of necessary antibodies and decreases the analysis time for pathologists. Furthermore, the possibility to select a particular area of the tissue allows performing a precise microdissection of the tissue of interest. However, TMA has some limitations which shares with conventional IHC methods. First of all, a complete pathological evaluation is not possible due to the small size of the tissue cores. For this reason it is important to select a representative spot of the examined lesion. Among the common shortcomings of TMA and IHC, the main one is the lack of reproducibility of the conventional scoring system of cell staining intensity (from 0 to 3+), which remains a subjective and time-consuming process, owing to intra- and inter-observer variability (108-110). Although automated IHC scoring systems, including both image software analyses and scanning hardware, have been developed (111, 112), their use in clinical practice still remains regarding. TMA technology does not replace routine histopathological assessment, but it is an unbiased and cost-effective method of data standardization, which is not possible in the routine.

### 3.3.2. EXTRACTION

FFPE tissues could not be treated with routine proteomic extraction methods, because of the extensive formaldehyde-induced cross-linking of proteins. However, in the last years advances in heat-induced antigen retrieval strategies, developed initially for IHC, have made varying degrees of cross-link reversal possible, thus enabling the use of such tissues for proteomic research (90, 92, 95, 113-118). All the reported studies identified the high concentrations of SDS, the exposure to high temperature and pH as necessary preconditions for protein cross-link reversal and restoration of immunoreactivity. In 2007, Becker et al, in collaboration with Qiagen (Germany), developed a targeted quantitative approach to extract full-length, immunoreactive proteins from formalin-fixed tissues. This method allows quantification of HER2 in breast tissue with immunoblotting and reversed-phase protein microarrays (91). In January 2011, Becker also proposed a new buffer system which allowed the extraction of proteins from over-fixed (over than 144 hours) or long-term stored tissues (for 20 years) (119). Commercial restrictions over buffer composition details, together with the high costs involved, prevented its widespread use and application in the
laboratory routine practice. Different groups including ours have developed protein extraction protocols based on Laemmli buffer containing SDS. This method allows a rapid, low cost and an efficient extraction of proteins from FFPE, even from aged paraffin blocks (42, 90). Once extracted, the protein lysate could be submitted to techniques, such as Western blot analyses, immunoprecipitation and reverse phase microarray approaches. The application of these methods to FFPE addresses the challenge of combining histopathology with molecular analysis and performing quantitative measurements. Nevertheless, protocol standardization and validation is required to ensure optimal extraction, identification and quantification of proteins for their application to routine clinical practice.

3.3.3. REVERSE PHASE PROTEIN MICROARRAY

Protein microarrays are an emerging class of nanotechnology assays to screen many different proteins simultaneously. They may be subdivided into arrays for protein profiling and arrays for functional studies (120-122). Arrays for protein profiling can be further divided into (a) forward and (b) reverse phase protein microarrays (FPPA and RPPA, respectively), depending on the way the sample is applied. In the case of the forward array setting, the protein lysate is analysed on a single microarray containing up to several thousands of different capture molecules, e.g., antibodies or aptamers. Here, many proteins of interest can be analysed in one sample during the same experiment. On the other hand, in the RPPA, hundreds or thousands of different protein lysates are immobilized onto one array. Using an appropriate ligand or antibody, one protein can be assayed in a large number of samples (60, 123). RPPAs have been applied for the identification of tumour-associated proteins and also for the identification of specific protein modifications. For example, RPPAs allow the examination of the activation state of crucial cellular pathways using antibodies against the total and the phosphorylated protein (66, 123). This method requires specific and high quality antibody against the protein of interest, which is usually evaluated by Western blotting.
3.3.4. ALTERNATIVE FIXATIVES

FFPE tissues are highly stable, cheap, and easily stored worldwide. The disadvantage of formalin – besides its toxicity (124) - is its cross-linking capacity (125) which hampers the use of FFPE tissue in molecular tests. Although progress has been made in analyzing proteins from FFPE samples, it is not surprising that alternative non-cross linking fixatives have been proposed to overcome the limitations of formalin. These fixatives include alcoholic and non-alcoholic solutions, such as: ethanol (126), methanol (127), acetone, the mixtures of fixatives Carnoy (128), Methacarn (129), the zinc-based fixatives (130), and the commercial fixatives FineFIX (131, 132), UMFIX (133), RCL-2 (134), Alcolin, HOPE (135), PAXgene (136). In order to substitute formalin, these fixatives should provide good tissue specimens for morphological examination, good immunoreactivity for immunohistochemical analysis and good macromolecule preservation for downstream molecular analyses. These are preliminary requirements. These alternative tissue fixatives have been examined and seem to be appropriate for DNA, RNA and protein analysis (42, 130, 131, 134, 137). In general, alcohol-based fixatives such as methacarn (138) or a combination of alcoholic fixatives and microwave treatment (43, 131) allow an improved preservation of macromolecules integrity, with a good morphological preservation. Although there are many alternative fixatives to formalin, none of these has hitherto replaced formalin in pathology departments. Maybe, only the banning of formalin by the European Union could stop pathologists from using it.

In our research, we wish to evaluate the potential application of molecular techniques in tissues treated with these kind of fixatives, by analysing morphology, IHC, DNA, RNA and proteins. To this end, we implemented a protein extraction protocol from tissues treated with formalin-free fixatives. The protein extracted through this method can be submitted to 2D-PAGE analyses, because the extraction buffer does not contain any ionic detergents that can modify the protein charge.
4. EXAMPLES OF CLINICAL APPLICATION OF PROTEOMICS METHODS IN ARCHIVE TISSUES

I explored the potentiality of protein analyses in archive tissues in two clinical research fields: the improvement of molecular diagnostic and prognostic tools and the identification of a new therapy target. The first one involved the validation of new antibodies directed to cervical cancer. The second one concerned molecular biology and proteomic study of RECQ1 helicase for the proposal of a new therapy target in glioblastoma.

4.1. CERVICAL CANCER

Cervical cancer is the second most common gynaecologic malignancy among women worldwide. Persistent HPV infection is the most frequent cause of this type of cancer. More than 150 types of HPV are known and are classified in groups according to the risk of cancer associated to the infection. However, it has been noticed that the high risk HPV distribution and the associated cervical cancer incidence depend on the geographical area (139). Worldwide epidemiological studies indicate that 15 different HPVs, namely 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82, are associated with cervical cancer, while three additional HPV types of the same genus can be classified as probable high-risk types, *i.e.* 26, 53 and 66 (140). HPV16 and HPV18 are the most frequently found in cervical cancer worldwide, being detected in approximately 50% and 20% of cases respectively (140, 141).

HPVs are small circular double-stranded DNA viruses that belong to the Papovaviridae family. The HPV genome is about 8 kb in length and encodes eight open reading frames, which are transcribed as polycistronic mRNAs (142). The HPV genome can be divided into three different regions: (1) a coding region containing the early genes, E1, E2, E4, E5, E6 and E7; (2) a region containing the late genes encoding the major (L1) and minor (L2) capsid proteins; and (3) a non-coding region, termed long control region (LCR), localized between open reading frames (ORF) L1 and E6, and containing most of the regulatory elements involved in viral DNA replication and
transcription (Figure 2). This genome structure is shared among different HPV genera, except for the E5 gene, which is not present in the majority of the beta types (the ones mainly infecting skin). HPV E6 and E7 genes are highly conserved in almost all HPV types, and in the case of cancer-associated HPV types they encode the major transforming proteins.

![Figure 2. HPV16 genome and functions of the early and late gene products (143).](image)

The life cycle of HPVs is tightly linked to the differentiation program of keratinocytes in the squamous epithelium (144). The production of viral particles occurs exclusively in suprabasal differentiated layers. After the infection, the genome is uncoated and transferred into the cell nucleus as a low copy number (20-100) episome. When these infected cells undergo cell division, the viral genome replicates and becomes equally segregated between the two daughter cells. One of the daughter cells migrates from the basal layer and initiates a program of differentiation. The other one continues to divide in the basal layer and provides a reservoir of viral DNA for further cell divisions. Since production of HPVs is restricted to suprabasal cells, those in the basal layer are not lysed by virion production, but continue to proliferate. This differentiation-dependence allows the infected cells to persist in the basal layers for several years. Differentiation of HPV-positive cells induces the productive phase of the viral life cycle, which requires cellular DNA synthesis.
machinery. The expression of E6 and E7 deregulates cell cycle control, thus pushing differentiating cells into S phase and allowing viral genome amplification in cells that normally would have exited the cell cycle. The late-phase L1 and L2 proteins encapsidate newly synthesized viral genomes, then virions are shed from the uppermost layers of the epithelium (Figure 3) (145, 146).

![Figure 3. The life cycle of HPVs (145).](image)

Mucosal HPVs preferentially infect the cervical transformation zone, which is the junction point of the endocervix columnar cells and the esocervix stratified squamous epithelial cells. The transformation zone epithelium is subjected to continuous changes during a woman’s life, and it has been suggested that the specific features of this region favour the development of cervical cancer. However, the majority of HPV infections do not lead to cytological anomalies or cancer, but they are cleared by the immune system in a relatively short time (6–12 months). A small number of infections persists and promotes the development of low- and high-grade cervical intraepithelial neoplasia (CIN), which may regress or progress to an invasive cervical carcinoma after a period of latency (147). There are additional risk factors which play a role in the progression of HPV-induced diseases, most likely by influencing the immune surveillance system or acting as additional risk factor. Among them, sexual habits, smoking, oral contraceptives, parity, host genetic predisposition and other viral infections have been reported (148-152).
In precancerous lesions, most HPV genomes persist in an episomal state, whereas in many high-grade lesions genomes are integrated into the host chromosome. Although no apparent hotspots have been identified, HPV integration often occurs near common fragile sites, which are naturally occurring regions of genomic instability (153, 154). The integration of viral DNA into the host genome is a crucial step to carcinogenesis. However, recent studies have suggested that in the development of high grade dysplasia HPV episomes may coexist with integrated copies of the genome (155). In lesions containing HPV episomes, the viral E2 protein directly represses early gene expression as part of a mechanism to regulate copy number. The integration of viral DNA disrupts E2 expression, leading to the deregulated expression of early viral genes, including E6 and E7. These proteins are critical in viral replication and induce proliferation, immortalization, and malignant transformation of the infected cells (156, 157). Both E6 and E7 are small proteins, approximately 18 and 13 kDa respectively, localized in the nucleus. The E6 proteins are also found in the cytoplasm and some studies have suggested that E7 has also a cytoplasmic component (158, 159). Several functions have been described for E6 and E7, which act synergistically in the development of HPV-induced cancers. The main activity of E6 is the degradation of the tumour suppressor protein p53 via the ubiquitin/proteasome pathway (160) and the pro-apoptotic protein BAK (161, 162). These events lead to resistance to apoptosis and increase in chromosomal instability. In addition, the activation of telomerase (163) and the postulated inhibition of degradation of SRC-family kinases by the E6 oncoprotein (164) seem to fulfill important functions in growth stimulation. A primary target of E7 is the retinoblastoma (Rb) family of proteins that control the activity of E2F transcription factors, key regulators of S phase genes (165). E7 inhibition of RB results in the upregulation of p16\textsuperscript{INK4A} (166, 167), in the stimulation of cyclins A and E (168, 169), and in the inactivation of the cyclin-dependent kinase inhibitors p21 and p27 (170, 171). Moreover, E6 and E7 induce genomic instability through multiple mechanisms, including aberrant centrosome abnormalities (172-174). E6 and E7 also target cytokine expression to modulate cell proliferation and interferon responses, contributing to immune evasion (175, 176). Figure 4 and Figure 5 show the main pathway affected by the two viral proteins.
Although most women will be infected with HPV at some time, the majority of acute HPV infections induce low-grade precursor lesions that regress spontaneously in more than 90% of cases. In only a minority of cases the virus infection persists and causes cell alterations leading to malignancy (177). Virus multiplication is confined to the nucleus. Consequently, infected cells exhibit a high degree of nuclear atypia. Koilocytosis describes a perinuclear clearing halo with a
pyknotic nucleus and is the characteristic feature of productive HPV infection (178). In a small percentage of cases, the persistency of the viral infection leads to the development of low grade disease, termed low-grade cervical intraepithelial neoplasia (CIN 1), which is characterized by abnormal differentiation in the lower third of the epithelium. The lesion may regress or progress to severe dysplasia (CIN 2, CIN2/3 and CIN3) or evolve to invasive cervical carcinoma. CIN1 lesions resemble productive infections while high-grade lesions such as CIN2 and CIN3 have a more extensive proliferative-phase, with the productive stages of the virus life cycle being only poorly supported.

Nowadays, cytological examination of smears (Pap test) in combination with colposcopy and HPV DNA testing are the most frequent methodologies in cervical cancer screening programmes. Although these technologies have dramatically reduced cervical cancer incidence and mortality, they present limitations and disadvantages in terms of sensitivity, specificity, inter- and intra-reproducibility (179). Only the HPV DNA test confirms HPV infection and defines the viral type. The current cytological and histological examinations cannot reliably distinguish the abnormal smears or biopsy which will progress to invasive cancer from the vast majority of cases that spontaneously regress. Moreover, the recognition of cervical dysplasia is still crucial in cases with borderline cytological and/or morphological features. In such cases, the histological diagnosis of cervical dysplasia lesions is highly subjective (180). A correct sub-classification of the squamous intraepithelial lesion (SIL) is needed to better stratify patients according to their real risk of developing cervical cancer, but also to avoid misclassification and overtreatment.

Immunological detection of HPV in human cells or tissues is often hindered for two main reasons: first, the late capsid proteins are only expressed in productive infections and second, the early proteins are usually expressed in small amounts in infected tissues. In addition, the production of specific antibodies to be used for immunochemistry has long been hampered due to the lack of a suitable in-vitro culture system to obtain HPV virions or antigens. To date, IHC detection of HPV
infection is performed by HPV L1 and surrogate biomarkers, such as p16\(^{INK4a}\), Ki67, hTERT and others (58, 167, 181-189).

### 4.2. ROLE OF RECQ HELICASES IN TUMOUR SUPPRESSION

Genetic information is stored in the base pairing of double stranded DNA (dsDNA). In many biological processes the complementary strands of the DNA duplex are separated to access the genetic information. The strand separation reaction is catalysed by DNA helicases. Helicases are ATP-dependent enzymes which disrupt the duplexes. Helicase-catalyzed DNA unwinding is a fundamental requirement for many aspects of nucleic acid metabolism, such as DNA replication, recombination, repair, transcription, translation and RNA splicing.

One of the most extensively studied groups of helicases is the RECQ family. In humans, five members of the RecQ family have been found: BLM, RECQ1 (also known as RECQL or RECQL1), RECQ4 (also known as RECQL4), RECQ5, and WRN (190-192). RECQ helicases are so-called ‘genome caretakers’ and do not seem to directly regulate tumourigenesis. It is likely that they are able to prevent genomic instability, and therefore their loss or inactivation would result in the accumulation of structural changes in oncogenes or tumour-suppressor genes leading to cancer (193, 194). Nevertheless, it is likely that RECQ helicases directly affect cellular transformation, influencing oncogene-induced senescence for example (195). Germline mutations in BLM, WRN or RECQ4, which are located on chromosomes 15q-26.1, 8p-12 and 8q-24.3 respectively, give rise to rare disorders associated with cancer predisposition, premature ageing and developmental defects. These disorders are Bloom's syndrome (BS), Werner's syndrome (WS) and Rothmund-Thomson syndrome (RTS) respectively. Remarkably, recent reports demonstrate that RECQ4 mutations are also responsible for two other apparently unrelated disorders: Rapadilino syndrome and Baller–Gerold syndrome (196) (197) (198-200). BLM and WRN helicases were also recently suggested to be involved in the promotion of tumour cell growth and proliferation (201, 202). Mutations in the RECQ1 and RECQ5 genes may be responsible for additional cancer
predisposition disorders, but this remains to be proven. Furthermore recent studies have associated a single nucleotide polymorphism of RECQ1 gene with reduced survival in pancreatic cancer patients (203, 204). A cancer specific role of RECQ1 is supported by two recent reports showing that silencing in cancer cells resulted in mitotic catastrophe and local and systemic administration of RecQL1-siRNA mixed with polyethyleneimine polymer or cationic liposomes prevented tumour growth in murine models (205, 206).

Biochemical studies have demonstrated that RECQ helicases unwind DNA with a 3’ to 5’ polarity in a variety of DNA structures other than standard B-form DNA duplexes. Consistent with the ability to unwind various DNA structures, several cellular functions have been attributed to RecQ proteins, including roles in stabilization and repair of damaged DNA replication forks, telomere maintenance, homologous recombination, and DNA damage checkpoint signaling (207-210).
AIM OF THE STUDY

The main goal of this PhD project is to define innovative proteomic methods and tools for the quantitative detection of biomarkers useful in clinical practice.

During the first year, the project was devoted to the development and optimization of protein extraction protocols and methods for fixed and paraffin embedded (FPE) tissues with different pre-analytical conditions. This part of the research was developed in the context of the European project IMPACTS work package on proteomics that aims at validating a commercial protein extraction protocol (QPProteome FFPE tissue kit) in formalin and FineFIX fixed tissues. This phase involved both pre-analytical treatment of tissues (tissue transport, fixation time, type of fixative) and analytical treatment of tissues (protein extraction, qualitative and quantitative protein detection) and was performed directly on clinical samples from surgical leftovers.

In order to explore the potential application of protein analyses on archive tissues, I analyzed two different aspects of clinical proteomics:

- the screening of non-surrogate biomarkers for cervical cancer useful for the early detection and for the progression risk assessment. This part was performed in collaboration with a company, which produces specific and high affinity monoclonal antibodies (MoAbs) directed against E7 HPV oncoprotein. The evaluation of this MoAbs was performed through IHC by comparing the staining efficiency with the following markers: the viral capsid protein L1, the surrogates p16, hTERT, p53, Ubiquitin and the proliferative marker Ki67.

- the identification of a new therapy target for glioblastoma, deepening the functions of human RECQ1 helicase. A combination of proteomic and molecular biology approaches was applied in this research. This phase of the project was performed in collaboration with the Genome Stability Laboratory at ICGEB, Trieste.

Hereafter is provided a synoptic table of methods used.
## Materials and Methods

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<th><strong>REVERSE PHASE PROTEIN ARRAY</strong> (paragraph 5.4, pag. 39)</th>
<th><strong>RNA INTERFERENCE</strong> (paragraph 6.1, pag. 40)</th>
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</thead>
<tbody>
<tr>
<td><strong>PROTEIN EXTRACTION</strong> (paragraph 6.2, pag. 40)</td>
<td><strong>SDS-PAGE AND WESTERN BLOTTING</strong> (paragraph 6.3, pag. 41)</td>
<td><strong>CELL CYCLE ANALYSES</strong> (paragraph 6.4, pag. 43)</td>
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<td></td>
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<td><strong>CLONOGENIC ASSAYS</strong> (paragraph 6.5, pag. 43)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>IMMUNOFLUORESCENCE</strong> (paragraph 6.6, pag. 44)</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS

1. SAMPLES COLLECTION

I used clinical samples that can be divided into three groups: (a) surgical leftovers, used for the parallel fixation trial (Formalin and FineFix) and protocol validation experiments; (b) independent clinical case studies from AT, used to study the candidate biomarkers; (c) human cancer cell lines used to assess and screen the validity of the biomarkers studied.

1.1. Surgical leftover tissues

Tissues were collected from three different hospitals within the IMPACTS group. In particular, 3 lung cancer specimens were supplied by the Institute of Pathology of Graz University, 3 colon cancer specimens were provided by the Department of Medical, Surgical and Health Sciences of Trieste University, and 3 breast cancer specimens were supplied by the Department of Biomedical Sciences and Human Oncology of Turin University. Each specimen was divided into two equivalent sets: one was fixed in 10% neutral-buffered formalin, paraffin embedded (FFPE) and dehydrated according to routine protocols for diagnosis; the other one was fixed in FineFix dehydrated, and paraffin-embedded, according to the manufacturer's recommendations. Samples were anonymized and their use was approved by the Ethic Committee of each above-mentioned University. The resulting FFPE and FineFix-paraffin embedded tissue blocks were stored at room temperature for future use. Hematoxylin-Eosin-stained sections were examined for each case by board certified pathologists to ensure similar degrees of cellularity and absence of necrosis. In order to compare the fixation procedures and the protein extraction efficiency among different tissues, protein lysates were prepared in five different university laboratories (Graz, Munich (TUM), Trieste, Turin and Verona). Four of them had received 72 sections and one only 9. A total of 81 lysates were then submitted to proteomic analyses. The study protocol was approved by the Ethic Committee of each above mentioned University.
1.2. Cervical cancer case study

Cervical cancer samples were provided by a multicentric retrospective cervical cancer case studies. The selection criteria were a diagnosis of cervical dysplasia or cervical invasive carcinoma. The study analyzed included 45 patients with a median age of 38.9 years (range = 19-79). The clinical diagnosis and the mean age at diagnosis were reported in following table (Table 1).

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>N.</th>
<th>Mean</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
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</thead>
<tbody>
<tr>
<td>CIN1</td>
<td>6</td>
<td>30.8</td>
<td>7.9</td>
<td>19</td>
<td>43</td>
</tr>
<tr>
<td>CIN2</td>
<td>8</td>
<td>33.9</td>
<td>6.4</td>
<td>20</td>
<td>39</td>
</tr>
<tr>
<td>CIN3</td>
<td>21</td>
<td>37.1</td>
<td>9.9</td>
<td>25</td>
<td>67</td>
</tr>
<tr>
<td>DYSPLASIA, NOS</td>
<td>1</td>
<td>26.0*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SQUAMOUS METAPLASIA</td>
<td>1</td>
<td>42.0*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CARCINOMA, NOS</td>
<td>1</td>
<td>32.0*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SQUAMOUS CELL CARCINOMA</td>
<td>7</td>
<td>59.6</td>
<td>16.4</td>
<td>32</td>
<td>79</td>
</tr>
</tbody>
</table>

* Individual age

Table 1. Clinical diagnosis of cervical biopsies used in the study. Abbreviations: SD, standard deviation; CIN, Cervical Intraepithelial neoplasia; NOS, not otherwise specified.

These specimens were characterized at clinical and molecular level for the presence and type of HPV using PCR-based systems or the Hybrid Capture assay (H2C, QIAGEN), during the clinical setting. Table 2 summarized the HPV types infection distribution.
Materials and Methods

Table 2. HPV type distribution among the 45 patients.

<table>
<thead>
<tr>
<th>HPV types</th>
<th>N. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV6</td>
<td>1</td>
</tr>
<tr>
<td>HPV16</td>
<td>17</td>
</tr>
<tr>
<td>HPV18</td>
<td>3</td>
</tr>
<tr>
<td>HPV31</td>
<td>3</td>
</tr>
<tr>
<td>HPV33</td>
<td>3</td>
</tr>
<tr>
<td>HPV52</td>
<td>1</td>
</tr>
<tr>
<td>HPV56</td>
<td>1</td>
</tr>
<tr>
<td>HPV58</td>
<td>1</td>
</tr>
<tr>
<td>HPV61</td>
<td>1</td>
</tr>
<tr>
<td>HPV66</td>
<td>1</td>
</tr>
<tr>
<td>HPV68</td>
<td>1</td>
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<tr>
<td>Multiple infections</td>
<td>7</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
</tr>
</tbody>
</table>

1.3. REQC1 case study

The protein expression profile of REQC1 was analysed in different type of tumours. Brain, colon, lung, and thyroid cancer tissues together with normal autopic heart tissue were provided by the Pathology Department of the Trieste University and submitted to IHC analyses of REQC1. From this test, REQC1 resulted to be a candidate biomarker for brain tumors. To deeply explore this hypothesis, 63 additional biopsies of glioblastoma were collected from the archive of the same pathology department. The selection criteria were: patients with a first glioblastoma diagnosed between 2003 and 2009. Among the 63 patients, 30 were males with a median age of 65 years (range 42-84) and 33 were females with a median age of 62 years (range 42-81). This case study was used to construct a TMA for the IHC analyses.
1.4. Cell lines

In this study the following cell lines of human origin were used:

- Glioblastoma cell lines: T98G (ATCC, CRL-1690) and U-87 (ATCC, HTB-14).
- Normal fibroblast cell line: IMR-90 (ATCC, CCL-186).

Cells were maintained in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with Glutamax (Life Tecnologies, Inc.), 10% (v/v) fetal bovine serum (FBS; Life Technologies, Inc.), 100U/ml penicillin and 100 µg/ml streptomycin and 2 µg/ml Gentamicine. Cells were grown at 37°C in an incubator supplied with 5% CO₂. Confluent monolayer cells were washed with phosphate-buffered saline (PBS) and treated with trypsin (0.25% (w/v) trypsin, 0.5 mM EDTA in PBS) at 37°C for 5 minutes or until cells were dispersed. An appropriate volume of complete growth medium was added and cells were pelleted. For sub-cultivation, cells were resuspended in complete growth medium, according to the following ratio:

- Glioma cells at a ratio of 1:6 to 1:8;
- Fibroblasts at 1:2 to 1:3.

Cells were frozen at a density of approximately 2 x 10^6 cells/ml in FBS with 10% DMSO and then stored at -80°C.

2. HISTOLOGICAL STAINING AND MICRODISSECTION

H&E staining was routinely performed on 3µm-thick sections (211).

Manual microdissection was performed to isolate tumour from perilesional tissue in cases where the amount of perilesional tissue was higher than 15% of the total amount. H&E stained section of each case was analysed by expert pathologists and the areas of interest were marked on the slide. Five consecutive unstained sections from the same tissue blocks were used for protein extraction (see the paragraph 5.1).
The H&E slides were also used to locate the dysplastic lesions and the tumour or the perilesional area on the original paraffin donor block for the construction of cervical cancer and glioblastoma TMAs.

3. TISSUE MICROARRAY CONSTRUCTION

For TMAs construction, selected areas were marked on the H&E slides. Tissue cylinders of 1.5 mm in diameter were taken from the selected regions of the donor paraffin block and were punched into a recipient paraffin block using a tissue-arraying instrument (Galileo TMA CK3500, Integrated Systems Engineering, Milano, Italy). Once completed, the TMA was placed upside-down onto a glass slide and incubated at 40°C for about 30 minutes in order to allow binding of the donor cores to the paraffin wax of the block. The aforementioned glass slide was used to level the block surface by gently pushing the cores into the block. After cooling, 4 µm thick sections were cut and mounted on microscope slides for subsequent H&E staining and IHC analyses.

The following TMAs were constructed in this study:

- Cervical TMAs, which comprise a total of 138 tissues cores. Multiple punches for each bioptic sample were done, taking different cervical intraepithelial lesions (from low grade to invasive squamous cancer) specifically selected by two expert pathologists (Table 3). Low grade lesions (LSIL) include normal epithelium with condyloma, condyloma and CIN1 dysplasia; high grade lesions (HSIL) consist of: CIN2, CIN2/3 and CIN3 displasias.
Materials and Methods

<table>
<thead>
<tr>
<th>Histopathological sub-classification</th>
<th>Number</th>
<th>Total number.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSIL</td>
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<td></td>
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<tr>
<td>Condyloma</td>
<td>64</td>
<td>95</td>
</tr>
<tr>
<td>CIN1</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>HSIL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN2</td>
<td>11</td>
<td>24</td>
</tr>
<tr>
<td>CIN2/CIN3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>CIN3</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>All lesions.</td>
<td></td>
<td>138</td>
</tr>
</tbody>
</table>

Table 3. Number of cervical lesions analyzed by TMAs.

- Glioblastoma TMA which comprises 82 tissues cores: 63 lesional and 19 perilesional tissue areas, specifically selected by an expert pathologist

4. IMMUNIHISTOCHEMISTRY

IHC staining was performed on 4 µm-thick tissue sections deposited on SuperFrost® Plus slides and incubated at least for 12 hours at 37°C. The antibodies used and the experimental conditions are listed on the table below. The immunostaining procedures were performed automatically by using the Benchmark device (Ventana Medical System) and manually by using heat-induced antigen retrieval method. For the manual detection, the incubations were performed in a humidified chamber. Briefly, tissue sections were deparaffinized, immersed in xylene for 30 minutes and then hydrated in a decreasing alcohol series. Endogenous peroxidase activity was blocked by incubating the tissue sections in 0.3% H₂O₂ for 20 minutes. The sections were subject to heat-
induced antigen retrieval solution\textsuperscript{2} for 20 minutes. To avoid unspecific binding, the sections were incubated for 20 minutes with blocking serum (Vectastain Universal Elite ABC kit, Vector Laboratories). Incubation with the primary antibody was performed for 1 hour as described in Table 4. Negative controls of sections without the primary antibody step were used. The slides were washed three times for 3 minutes each in PBS and 0.1% of Triton X-100, and incubated for 60 minutes with the biotinylated secondary antibody and for 30 minutes with Vectastain ABC system. Detection was made using the DAB substrate kit (Vector Laboratories) for 8-10 minutes. The sections were counterstained with Mayer hematoxylin. Double IHC of Ki67 and RECQ1 was performed using DAB and FastRed detection system and the Benchmark device (Ventana Medical System).

Only for ubiquitin, DAKO LSAB+/HRP kit (Dako Cytomation) was used for staining. After the deparaffinization and the inhibition of the endogenous peroxidase activity, the tissue sections were blocked with 3% BSA for 20 minutes. The primary antibody was diluted in 5mM Tris-HCl with 1% BSA and incubated for 1 hour at room temperature. The slides were washed and then incubated with the biotinylated antibody and with the streptavidin-HRP solution as previously described. Detection was made using the DAB chromogen solution provided by the kit for 5 minutes. The sections were counterstained with Mayer hematoxylin as usual.

Cells showing a positive staining were counted across three high power fields (HPF) and making the average. The staining intensity was evaluated by a score from 0 to 3+, at cytoplasmatic and nuclear level, across three HPFs.

\textsuperscript{2} See Table 4 for the antibody-specific antigen retrieval method used.
### Materials and Methods

<table>
<thead>
<tr>
<th>Antibody (Clone)</th>
<th>#Order</th>
<th>Localization</th>
<th>AR</th>
<th>Dilution</th>
<th>IT</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>#2232</td>
<td>Membrane</td>
<td>10 mM Buffer Citrate pH 6</td>
<td>1:150</td>
<td>RT</td>
</tr>
<tr>
<td>E-cadherin (36)</td>
<td>#610181</td>
<td>Membrane</td>
<td>10 mM Buffer Citrate pH 6</td>
<td>1:2000</td>
<td>RT</td>
</tr>
<tr>
<td>Ki67 (30_9)</td>
<td>#790-4286</td>
<td>Nucleus</td>
<td>100 mM Tris-Borate 1 mM EDTA pH 8.0</td>
<td>Pre-diluted</td>
<td>RT</td>
</tr>
<tr>
<td>p16 (F-12)</td>
<td>#sc-1661</td>
<td>Nucleus/Cytoplasm</td>
<td>10 mM Buffer Citrate pH 6</td>
<td>1:150</td>
<td>RT</td>
</tr>
<tr>
<td>p53 (D07)</td>
<td>#800-2912</td>
<td>Nucleus</td>
<td>10 mM Buffer Citrate pH 6</td>
<td>Pre-diluted</td>
<td>RT</td>
</tr>
<tr>
<td>hTERT (Y182)</td>
<td>#ab32020</td>
<td>Nucleus/Cytoplasm</td>
<td>10 mM Buffer Citrate pH 6</td>
<td>1:100 37°C</td>
<td></td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>#Z0458</td>
<td>Nucleus</td>
<td></td>
<td>1:500</td>
<td>RT</td>
</tr>
<tr>
<td>L1</td>
<td>#VAHP50</td>
<td>Nucleus/Cytoplasm</td>
<td>10 mM Buffer Citrate pH 6</td>
<td>Pre-diluted</td>
<td>RT</td>
</tr>
<tr>
<td>RQ-CT³</td>
<td>Sigma Genosys</td>
<td>Nucleus</td>
<td>10 mM Buffer Citrate pH 6</td>
<td>1:150</td>
<td>RT</td>
</tr>
<tr>
<td>RQ-FL⁴</td>
<td>home made (212)i</td>
<td>Nucleus</td>
<td>10 mM Buffer Citrate pH 6</td>
<td>1:150</td>
<td>RT</td>
</tr>
<tr>
<td>GFAP (EP672Y)</td>
<td>#760-4345</td>
<td>Cytoplasm</td>
<td>100 mM Tris-Borate 1 mM EDTA pH 8.0</td>
<td>Pre-diluted</td>
<td>RT</td>
</tr>
</tbody>
</table>

Table 4. Antibodies used for IHC analyses. Abbreviations: AR, antigen retrieval; IT, Incubation Temperature; RT, room temperature.

³ The custom rabbit polyclonal anti-RECQ1 antibody against the C-terminal region of RECQ1 (RQ-CT) was raised against a 16 amino acid peptide corresponding to residues 634-649 of RECQ1 (C-SGSKNTGAKKRKIDDA) with an N-terminal cysteine conjugated to the keyhole limpet hemocyanin (KLH) carrier protein.

⁴ The rabbit polyclonal anti-RECQ1 antibody against the full-length RECQ1 (RQ-FL) was made by injecting the rabbits with full-length recombinant RECQ1 expressed in insect cells.
Specificity of RECQ1 antibody was assessed by preadsorption test. Before the incubation with primary antibody, the RECQ1 antibody and the specific immunizing peptide have been incubated in a molar ratio of 1:5 at 37°C for 30 minutes.

The screening of the 39 MoAbs against the HPV oncoviral protein was made by using the Vectastain Universal Elite ABC kit (Vector Laboratories) as previously described, but blocking of unspecific sites was performed with 3% BSA. No antigen retrieval procedures were performed. Information about staining conditions are confidential. Specificity of the selected MoAb was tested by preadsorption assay as described for RECQ1.

5. PROTEIN ANALYSES IN FPE TISSUES

5.1. Protein extraction from FFPE and FineFix tissue sections

In order to compare the fixation procedures and the protein extraction efficiency among different tissues, protein lysates were prepared in five different laboratories (Graz, Munich, Trieste, Turin and Verona) from matched FFPE/FineFix lung (Graz), colon (Trieste) and breast (Turin) cancer samples. A flowchart of the study is provided in Figure 6.

For each case an H&E stained slide with the tumour area marked and five consecutive 10 µm thick unstained sections were cut, mounted onto glass slides and distributed to the participants. Tissue slides were sent at room temperature. Protein extraction was performed in each laboratory using the Qproteome FFPE Tissue Kit (QIAGEN) as previously reported for FFPE (91) and FineFix (213) tissue sections. Tissue sections were deparaffinized in xylene and rehydrated in graded ethanol series (100%, 96% and 50%); each step was performed twice for 10 minutes at room temperature. Tumor areas were scratched from the unstained slide with a needle. The microdissected tissue was transferred into the adequate volume of Qproteome FFPE Tissue Kit Buffer (100 µl for two slides of about 0.5 cm in diameter) and extraction was performed according to the manufacturer's recommended procedure. Protein lysates were stored at -20°C.
5.2. Protein extraction from FFPE cervical tissues

This protocol describes a rapid and low-cost method based on SDS-containing-Laemmli buffer which allows an efficient extraction of proteins from FFPE (46, 92). About 3 sections up to 10 µm thickness (~ 25 mm² each) were cut and placed in a 1.5 ml collection tube. Tissues were deparaffinized in 1 ml xylene (2 x 5 minutes) and rehydrated in graded ethanol series (2 x 100%, 90%, 70%, 5 minutes). Tissue pellets were resuspended in 150 µl of freshly prepared Laemmli Buffer (100 mM Tris-HCl in distilled water, pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 4% (v/v) β-mercaptoethanol) and incubated at 99°C for 20 minutes. Tubes were spin down at maximum speed for 5 minutes at 4°C. Staining solution (0.02% of Bromophenol blue stock solution 1% (w/v) Bromophenol blue, 50 mM Tris-base) was added to the supernatant. The lysates were stored at -20°C.
5.3. Western blotting in FPE tissues

The protein lysates (1/10 of the total protein extract volume) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were heated at 95°C for 5 minutes in 5X SDS-sample buffer (250 mM Tris-HCl, pH 6.8, 500 mM DTT, 10% SDS, 0.5% bromophenol blue, 50% glycerol), spun down and then analyzed by SDS-PAGE, according to the standard Laemmli method (214). Gels were run in SDS electrophoresis buffer (25 mM Tris, 190 mM glycine, 0.1% SDS).

After electrophoresis, the proteins were transferred onto a PVDF membrane. The electro-transfer was performed in transfer buffer (96 mM glycine, 12 mM Tris, and 10% methanol) at 4°C for 1 hour at 350 mA. Membranes were incubated with the primary antibodies (Table 5) after blocking. Then the membranes were washed four times with PBST (PBS with 0.1% Tween 20) for 5 minutes and were incubated with the proper HRP-conjugated secondary antibodies (from Pierce, diluted 1:10000 in blocking solution). Washing was performed 5 times with PBST for 5 minutes. Incubation with the chemiluminescent reagents (Amersham Biosciences ECL Western Blotting Detection Reagents or Millipore Immobilon™ Western Chemiluminescent Substrates) was performed according to the manufacturer’s instructions. Immunoblots were developed on a Kodak Biomat XAR film.

See Table 5.
## Materials and Methods

### Antibodies used for Western blotting and reverse phase array analyses

<table>
<thead>
<tr>
<th>Primary Antibody (clone)</th>
<th>MW (kDa)</th>
<th>#Order</th>
<th>Blocking</th>
<th>Primary Antibody Dilution</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin (AC15)</td>
<td>42</td>
<td>#A5441 (Sigma)</td>
<td>5% MP/PBST</td>
<td>1:10000</td>
<td>5% MP</td>
</tr>
<tr>
<td>HER2/NEU</td>
<td>185</td>
<td>#A0485 (Dako Cytomation)</td>
<td>5% MP/PBST</td>
<td>1:500</td>
<td>PBST</td>
</tr>
<tr>
<td>EGFR</td>
<td>170</td>
<td># 2232 (Cell Signaling)</td>
<td>5% BSA/PBST</td>
<td>1:2000</td>
<td>5% BSA</td>
</tr>
<tr>
<td>E-cadherin (36)</td>
<td>120</td>
<td>#610181 (BD Biosciences)</td>
<td>5% MP/PBST</td>
<td>1:5000</td>
<td>5% MP</td>
</tr>
<tr>
<td>ER (578-595)</td>
<td>67</td>
<td>#E0646 (Sigma)</td>
<td>0.5% Casein/PBS</td>
<td>1:3000</td>
<td>0.5% Casein/PBST</td>
</tr>
<tr>
<td>MoAb oncoviral protein</td>
<td>30</td>
<td>Confidential information</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Antibodies used for Western blotting and reverse phase array analyses. Abbreviations: MW, molecular weight; MP, nonfat dry milk powder; BSA, bovine serum albumin. PBST, PBS with 0.1% Tween 20.
5.4. RPPA of protein lysates from FFPE and FineFix tissues

For every protein lysate, a 5-fold dilution series plus a negative control consisting only of QproteomeFFPE Tissue Kit buffer (undiluted, 1:2, 1:4, 1:8, 1:16, buffer) was prepared and transferred to a 96-well plate. Protein lysate microarrays (or reverse phase protein microarrays) were generated by using the Bio-Rad Calligrapher (BioRad, Hercules, CA). For each dilution series, 3 replicates were applied onto a nitrocellulose coated glass slide (FastSlide, Whatman/Schleicher and Schuell). For protein detection, the slides were incubated in a peroxidase blocking solution (DAKO) for 1 hour, followed by blocking in 5% milk powder/PBST for 1 hour. Primary antibodies reacting with β-Actin, E-cadherin, EGFR, HER2, or ER (Table 5) were applied at 4°C for about 16 hours. After incubation with the horseradish peroxidase-coupled secondary antibodies (21°C; 1 hour), specific binding was detected using the ECLPlus and ECLadvance Western Blot Detection System (GE Healthcare). For estimation of total protein amounts, arrays were stained in parallel with Sypro Ruby Protein Blot Stain (Molecular Probes) according to the manufacturer’s instructions and visualized on an Eagle Eye (Stratagene). The developed films were scanned individually (MP190, Canon). Intensities of protein spots were measured using the MicroVigene software package (VigeneTec) as described elsewhere (215). Relative expression levels of proteins were calculated by normalization to total protein amount. Antibodies used for the protein lysate microarrays were previously validated for specificity by Western blot analysis with tissue lysates.
6. PROTEIN ANALYSES ON CELL LINES

6.1. RNA interference

Transient transfection was performed by using a pool of 4 siRNAs against RECQ1 (NM_032941: Dhamacon-SMARTpool) (Table 6) and the Hyperfect transfection system (QIAGEN), following the manufacturer's instructions. The siRNA pool was prepared at a final concentration of 100 nM and mixed with the HiPerFect Transfection Reagent, according to the manufacturer's instructions. The samples were incubated for 10 minutes at room temperature (15–25°C) to allow the formation of transfection complexes. Then, the complexes were added to the cell suspension and 0.25x10⁶ of T98G, U87 and IMR90 cells per well of 6 wells plates were seeded. The cells were incubated under normal growth conditions (37°C and 5% CO₂) for 72 hours. RNAi control experiments were performed using a duplex siRNA against Luciferase (Dharmacon).

<table>
<thead>
<tr>
<th>Name</th>
<th>Target sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence 5</td>
<td>CUACGGCUUUGGAGAUAAU</td>
</tr>
<tr>
<td>Sequence 6</td>
<td>GAUUUAUAGGCACUUGGUA</td>
</tr>
<tr>
<td>Sequence 7</td>
<td>GGGCAAGCAUGAAUUGUAGA</td>
</tr>
<tr>
<td>Sequence 8</td>
<td>GAGCUUAUGUUACCAGUU</td>
</tr>
</tbody>
</table>

Table 6. Individual sequence of siRNA against RECQ1 from Dhamacon SMART pool (NM_032941)

6.2. Protein extraction

To prepare whole cell extracts, 90% confluent cells were washed with PBS, trypsinized, washed with cold PBS and lysed.

T98G, U-87, and IMR-90 whole cell extracts were prepared in HNNG lysis buffer (15 mM Hepes pH 7.5, 250 mM NaCl, 1% (v/v) NP-40, 10% (v/v) glycerol, 1 mM PMSF) supplemented with 0.2
mM sodium orthovandate (Sigma), 10 mM sodium glycerol-2-phosphate (Sigma), 25 mM NaF (Sigma) and protease inhibitors cocktail tablets (Roche).

Cells suspension were sonicated 3 times for 10 seconds, avoiding bubbles formation and excessive heating of the solution. Then samples were clarified by centrifugation at 14,000 x g for 10 minutes at 4°C. Protein concentrations were estimated by Bradford protein assay (Bio-Rad). A standard curve was obtained through serial dilutions of BSA in the specific lysis buffer. Aliquots of the extracts were stored at -80°C, and operative aliquots was maintained at 4°C.

6.3. SDS-PAGE and Western blotting analyses

The whole protein lysates (equals to 10 µg of proteins) were separated on 12% SDS-PAGE, according to the previously described protocol (see paragraph 5.3). After electrophoresis run, gels were stained by Coomassie Brilliant Blue staining (0.2% Coomassie Blue R-250, 25% methanol, 10% glacial acetic acid) for 30 minutes, and then destained with Coomassie destaining solution (25% methanol, 10% glacial acetic acid).

After electrophoresis, the proteins were transferred onto a PVDF membrane, following the protocol described on paragraph 5.3. The primary antibodies used and the incubation conditions are reported in Table 7. Membranes were detected by immunoblotting using the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) or Immobilon™ Western Chemiluminescent Substrates (Millipore). Subsequent immunoblotting were performed on the same membranes after stripping in 100 mM β-mercaptoethanol, 62.5 mM Tris-HCl pH 6.8, 2% SDS for 30 minutes at 55°C and washing twice with TBST 10 minutes. Immunodetections were performed as above described from incubation with blocking solution.
### Primary Antibody (clone)

<table>
<thead>
<tr>
<th>Primary Antibody (clone)</th>
<th>MW (kDa)</th>
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<th>Blocking</th>
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<th>Secondary Antibody</th>
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<td>β-Actin-HRP conjugated (AC15)</td>
<td>42</td>
<td>#A3854 (Sigma)</td>
<td>5% MP/PBST</td>
<td>1:20000 5% MP/PBST</td>
<td>Mouse</td>
</tr>
<tr>
<td>α-Tubulin (B-5-1-2)</td>
<td>50</td>
<td>#T6074 (Sigma)</td>
<td>5% MP/PBST</td>
<td>1:10000 5% MP/PBST</td>
<td>Mouse</td>
</tr>
<tr>
<td>RECQ1 (H-110)</td>
<td>70</td>
<td>#sc-25547 (Santa Cruz Biotecnology)</td>
<td>5% MP/PBST</td>
<td>1:2000 5% MP/PBST</td>
<td>Rabbit</td>
</tr>
<tr>
<td>RAD51 (H-92)</td>
<td>35</td>
<td>#sc-8349 (Santa Cruz Biotecnology)</td>
<td>5% MP/PBST</td>
<td>1.1000 5% MP/PBST</td>
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</tr>
<tr>
<td>γ-H2AX pSer139 (JBMW301)</td>
<td>14</td>
<td>#05-636 Upstate (Millipore)</td>
<td>5% MP/PBST</td>
<td>1.1000 5% MP/PBST</td>
<td>Mouse</td>
</tr>
</tbody>
</table>

Table 7. Antibodies used for Western blotting analyses.
6.4. Cell cycle analyses

Cell cycle profile distributions of T98G and U87 cells were performed on transiently siRNA transfected cells 72 hours after transfection. Cells were pulsed for 1 h with BrdU (Sigma) at a final concentration of 10 μM. BrdU positive cells were detected by using a mouse anti-BrdU-FITC primary antibody followed by an anti-mouse Alexa 488-conjugated secondary antibody. Cells were collected and analyzed by flow cytometry on a FACSCalibur (Beckton Dickinson) to simultaneously determine the cell-cycle profile (DNA content) by incorporation of propidium iodide and the S-phase cell population by incorporation of BrdU. Cell cycle profile distributions were determined with the CellQuestPro and Modfit LT 3.0 software.

6.5. Clonogenic assays

Colony forming assays were made in vitro as already described (216). Assays were performed in six-well plates, with clones produced either by transfection with a luciferase-siRNA duplex as control or with a RECQ1-siRNA. Cells were seeded at different dilutions (100, 200, 400, 800, 1600 and 3200 cells per well) in six-well plates and incubated under normal growth conditions (37°C and 5% CO₂). After one week, the medium was removed and the cells were rinsed carefully with PBS. Formed colonies were fixed and stained in 3 ml of a mixture containing 6.0% glutaraldehyde and 0.5% crystal violet for at least 30 minutes. After glutaraldehyde and crystal violet removal, plates were carefully rinsed in water. The plates with colonies were dried at room temperature, before colonies count. The counting was done using VersaDoc 4000 imaging system (BioRad). Proliferative capacity of control cells and of the RECQ1-depleted cells was evaluated by their plating efficiency (PE) and expressed as colony forming capacity. The PE was calculated as the average ratio of the number of formed colonies versus the number of cells seeded and was expressed as percentage. Cell survival assays were performed by plating transfected cells before treatment with hydroxyurea (HU, Sigma) or temozolomide (TMZ, Sigma). Before seeding, cells
were treated overnight with different doses of HU (0.2, 2, 10 and 20 mM) or TMZ (5, 50, 250 and 500 µM). Surviving fractions were calculated following a procedure already published (216).

**6.6. Immunofluorescence**

Immunofluorescence was performed as already described (217). 20x10³ cells were seeded in chamber slides (NALGENE) after transient downregulation of the RECQ1 expression by RNA interference for 24 hours. A second transfection with the siRNA was repeated immediately after the cells were seeded in the chamber slides. The cells were left under these conditions for 72 hours. Then the medium was removed and the chamber slides were washed with PBS. Cells were then fixed with 3.5% paraformaldehyde in PBS for 20 minutes at room temperature, quenched with 125 mM of glycine solution for 5 minutes at room temperature and washed four times with PBS. The cells were permeabilized for 5 minutes with 0.2% Triton X-100 in PBS and treated thrice with 1% BSA in PBS 0.2% Tween-20 (PBST) buffer for 10 minutes each to block unspecific binding of antibodies. The cells were then incubated with different antibodies for 1 hour at 37°C and washed thrice for 5 minutes each at room temperature (Table 8). The cells were incubated for 1 hour at 37°C with the FITC conjugate anti-mouse secondary Alexa 488 and anti-rabbit secondary Alexa 594 antibodies (Invitrogen Molecular Probes). After three washes in PBS, cells were treated with RNase A. Nuclei were counterstained with Toto3 (Invitrogen Molecular Probe), diluted 1:20000 in 0.2% Triton X-100 in PBS. Coverslips were mounted with Vectashield (Vector Laboratories).

BrdU staining was performed after incubation of cells in 10 µM BrdU (Sigma) for 1 hour and DNA denaturation by 2N HCl for 30 minutes at 37°C and then followed by permeabilization.

Confocal fluorescence analysis was performed on a Zeiss LSM 510 Meta confocal microscope. Images were acquired using the LSM software. The cells containing γ-H2AX and RAD51 foci were counted in at least 100 nuclei.
Table 8. Antibodies used for Immunofluorescence analyses.

7. STATISTICS

7.1. Extraction protocol validation statistics

In order to avoid the multiple testing problems arising from analysing different fixation methods with antibody measurements, we adapted Goeman’s global test (218). Therewith it was possible to test whether any of the fixation methods caused any difference in protein lysate microarray measurements by one single $p$-value. Each measurement was rank transformed in order to reduce the influence of outliers. Furthermore, for each specimen the mean rank was subtracted in order to remove the block effect of the specimens. The global test was applied by using the permutation test criterion with 10000 replications. Pair wise differences between fixation methods were tested by Wilcoxon’s signed rank sum test. Values of $p$ below 0.05 were considered statistically significant. Analyses were performed through R 2.9.0 (http://www.r-project.org/) and R-package globaltest 4.10.0 provided by the Department of Medical Statistics of the Georg-August-University at Göttingen, Germany (http://www.ams.med.uni-goettingen.de/de/sof/ld/rd/ld.f1.r).
7.2. Cervical cancer TMAs statistics

For E7 antibody and the other biomarkers, one-way analysis of variance was performed to detect significant differences in the mean number of positive cells and in the mean staining intensity (evaluated by a score from 0 to 3+), at cytoplasmatic and nuclear level, among different types of cervical lesion, by epithelial layer.

We made use of repeated measures analysis of variance to ascertain significant differences in the mean number of positive cells between E7 and each biomarker. Moreover, Spearman’s correlation test and linear regression analysis were employed in order to detect correlations between E7 and the other biomarkers. Two sided \( p \)-value less than 0.05 was considered statistically significant. Analyses were performed with Stata SE 9.2 software (Stata Corp College Station, TX, USA).

7.3. Glioblastoma TMA statistics

The Wilcoxon’s test for matched-samples was employed to evaluate differences in IHC analyses between tumour tissues and normal peri-lesional tissues. IHC measures of outcome were: the proportion of positive cells for RECQ1, the intensity of the signal and the number of positive cells for Ki67. Spearman’s correlation test was applied to assess the relationship between intensity score of RECQ1 and the number of positive cells in lesional tissues. Kruskal-Wallis test was applied to investigate differences in the percentage of positive cells for Ki67 and RECQ1 among groups defined by patient gender and the intensity score of RECQ1. Two-sided \( p \)-value less than 0.05 was considered statistically significant. Analyses were performed with Stata SE 9.2 software (Stata Corp College Station, TX, USA).
RESULTS

My research activity was devoted to explore the potential of conventional and innovative proteomic approaches in clinical cancer research on AT. The first part of my project focused on the standardization of protein extraction protocols from FPE tissues and the validation of quantitative protein analysis. This part was strictly connected with the European project IMPACTS. In order to investigate the possible application of clinical proteomics in AT, two different fields were explored: (a) the screening of new non-surrogate biomarkers for cervical cancer useful for the early detection and for the progression risk assessment; (b) the identification of a new therapy target for glioblastoma, deepening the functions of human RECQ1 helicase. These two studies were performed in collaboration with a company and with the Genome Stability Laboratory (ICGEB, Trieste) respectively.

1. STANDARDIZATION OF PROTEIN ANALYSIS OF TISSUES FIXED WITH FORMALIN AND FINEFIX (132)

The aim of the IMPACTS work-package on proteomics was to compare protein expression in matched lung, colon and breast cancer tissues fixed with formalin and FineFix. Tissue samples were fixed either in formalin (FFPE) or FineFix (Milestone) in three different European pathology departments (Graz, Torino and Trieste). The tissue slides were inspected by histology and five unstained sections from three matched samples of each tissue were distributed within the IMPACTS consortium. The comparison of protein recovery efficiency was evaluated by Western blot and RPPA technology. IHC analysis was performed in order to compare morphology and immunoreactivity in tissues treated with different pre-analytical conditions.
1.1. Protein extraction

Five institutions (Graz, Munich, Torino, Trieste and Verona) were involved in the protein extraction validation from matched FFPE/FineFix cancer tissue samples. Four groups participated in the validation of protein extraction from FineFix specimens. In order to avoid variation unrelated to the fixative used, each group extracted proteins by employing the same method. Typically, 100 µl extraction buffer were used for protein solubilization from FFPE and FineFix tissue sections. All the five groups successfully extracted proteins from FFPE and FineFix tissues. Western blot analysis against β-Actin was performed to demonstrate successful protein extraction and suitability for downstream protein lysate microarray analysis. E-cadherin, EGFR and ER were also detected by Western blot. Representative Western blot detection of the analyzed proteins is shown in Figure 7 and 9.

![Western blot analysis](image)

**Figure 7.** Representative β-Actin Western blot in FFPE and FineFix fixed lung, colon, and breast cancer samples.
Figure 8. Representative Western blot analyses against β-Actin, EGFR and E-cadherin in matched FFPE and Finefix colon cancer tissues.

1.2. Quantitative protein analysis

Several studies have proven that RPPA is a powerful technology for quantitative protein analysis of routinely processed FFPE tissues (91, 98, 219). For this reason, this methodology was used to quantitatively measure protein abundances in tissue lysates. Twelve protein lysate microarrays were printed from 25 µl of starting material for each case. In addition, pools of lysates from the samples were also spotted on the same slides. For each case three replicates and a 6-fold dilution series were spotted; thus for each case 18 spots are incorporated into the calculations. This array analysis equals 2160 Western blot lanes: 12 cases (including 3 pools) x 18 spots x 5 antibodies x 2 cohorts (FFPE/FineFix). This number indicates the high throughput potential of the protein microarray technology.
These protein microarrays were probed with antibodies against β-Actin, E-cadherin, EGFR, HER2, and ER (Figure 9). Following antibody-based protein detection, the developed films were scanned and analyzed with a specific software, as described in Materials and Methods (paragraph 5.4). In order to compare intensity values between FFPE and FineFix, the average pixel intensity of each spot was normalized to the intensity values obtained for the total protein and detected by SyproRuby stain. The global comparison of the intensity values (including all five antibodies) showed that FineFix enhanced the antigen or protein preservation for the selected antibody panel ($p = 0.04$), in comparison to the matched FFPE samples. The pair-wise protein comparison between the fixatives (for each antibody) indicated differences in the signal intensity for EGFR ($p = 0.007$) and HER2 ($p = 0.02$) in the FineFix samples compared to the FFPE ones as shown in Figure 9. In contrast, E-cadherin, ER, and β-Actin signal intensities did not show significant
differences ($\rho > 0.05$) between FFPE and FineFix (Figure 10). Overall, we observed a modest increase for E-cadherin in the FineFix samples, whereas $\beta$-Actin signals were slightly increased in FFPE samples, but these changes did not reach statistical significance.

Figure 10. Box plot of the pair-wise proteins comparison between the fixatives.
1.3. IHC analyses

IHC staining was performed to evaluate the suitability of FineFix in maintaining tissue morphology and antigen immunoreactivity. The analyses were performed by the same institutions which provided the tissues for validation analyses. IHC analyses of the selected BMs showed comparable results between formalin and Finefix treated samples. FinFix tissues provide good tissue histology for the morphological examination and good immunoreactivity for IHC analyses, as shown in Figure 11.

![Representative IHC staining against E-cadherin and EGFR on matched colon cancer tissues fixed with formalin and FineFix.](image)

Figure 11. Representative IHC staining against E-cadherin and EGFR on matched colon cancer tissues fixed with formalin and FineFix.
2. HPV MoAbs VALIDATION

2.1. MoAbs screening

Thirty-nine monoclonal antibodies (MoAbs) directed against the E7 viral oncoprotein were supplied by the company. The screening was performed in a TMA that comprise 12 LSILs, 12 HSILs, 6 squamous carcinoma. All the specimens were HPV16 positive at genotyping. This first analysis aimed at a qualitative evaluation of the specificity of the antibodies for the cervical lesions. Table 9, displays the staining status of cervical lesions for each antibody clone. The battery of MoAbs showed different specificities for the cervical lesions, suggesting their possible utility in differential diagnosis. In detail, 18 MoAbs were positive for all the 30 cervical lesions analyzed, while 9 were negative for all the lesions, suggesting that these antibodies could be strain specific. Eight MoAbs were negative in the HSIL, 3 were negative in the squamous invasive carcinoma, while the MoAb35 recognize only LSIL. For all the MoAbs analyzed, the HPV oncoprotein expression was found both in the cytoplasm and in the nucleus, indicating that this protein is involved in different cellular pathways and could have different post-translational modifications. Figure 12 shows examples of the different IHC staining by the MoAbs.
Table 9. Summary results of MoAbs screening (the numbers indicate the different MoAb clones; blue cells represent positive stains).
Figure 12. Representative IHC staining of the different group of HPV MoAbs (O.M. 20X). Abbreviations: SCC, squamous cell carcinoma.

2.2. Specificity analyses of the MoAbs

The specificity of the MoAbs was confirmed by pre-adsorption tests. Figure 13 shows that only a minimal non-specific stain can be detected after pre-adsorption of the tissue with the specific viral peptide.
Results

Figure 13. Pre-adsorption test using the specific oncoviral peptide on cervical cancer tissues. Immunostaining with the E7 antibody in cervical tissue (1) and after preadsorption with the specific peptide (2) (O.M. 40X).

Protein extraction from representative FFPE cervical biopsies were performed in order to confirm the specificity of the E7 antibody. The E7 protein was successfully detected, obtaining a clear signal at the right molecular weight (~30 kDa). The β-actin western blot analyses was also performed to demonstrated the efficiency of the extraction (Figure 14).

Figure 14. E7 and β-actin Western blot analyses in FFPE cervix tissues.
The aim of this part of my project was to compare the new E7 antibody with the following biomarkers: the HPV capsid protein L1, p16, hTERT, p53, Ubiquitin (Ub) and the proliferative marker Ki67.

2.3. IHC comparison between E7 and the other biomarkers

Once the antibodies passed our screening, it was chosen the molecule that gave better results in terms of staining and specificity. The selected molecule was validated with other TMAs, which comprised a total of 138 tissues cores from different cervical intraepithelial lesions and with different HPV types infections.

In this study, I analyzed 138 squamous cervical lesions, from low grade dysplasia to invasive cancer. In particular, I analyzed 95 LSILs, which comprised 64 condylomas and 31 CIN1 lesions, 24 HSILs, which consisted of 11 CIN2, 4 CIN2/CIN3 and 9 CIN3 lesions, and 19 squamous cell carcinomas.

HPV DNA was found in the 73% of LSILs, in the 96% of HSILs, and in all squamous cell carcinomas. Multiple HPV infections was found in 6% of LSILs, 23% of HSILs and in 3% of squamous cell carcinomas. HPV 16 was found in all the 14 lesions characterized by multiple infections. Table 10 provide a summary of the HPVs typing distribution by lesion.
## Results

Table 10. Summary table of HPV typing distribution by lesions.

<table>
<thead>
<tr>
<th>Virus</th>
<th>LSIL Single infection</th>
<th>LSIL Multiple infection</th>
<th>HSIL Single infection</th>
<th>HSIL Multiple infection</th>
<th>Squamous cell carcinoma Single infection</th>
<th>Squamous cell carcinoma Multiple infection</th>
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<td>10</td>
<td>16</td>
<td>7</td>
<td>97</td>
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</table>
2.3.1. E7

For the evaluation of E7 staining, cytoplasmatic and nuclear immunoreactivity was evaluated, as number of staining positive cells. On a total of 138 cervical squamous lesions, we were not able to evaluate 45 due to either non-representative arrayed areas or lost spot during the process of cutting or staining. In the 64 LSILs, the mean number of staining positive cells was 58.3 (SD = 21.0), in the 15 HSILs it was 62.7 (SD = 21.2). In the 14 squamous cell carcinomas it was 40.3 (SD = 28.4). Analysis of variance showed significant differences in the mean value of positive staining cells between LSIL and squamous cell carcinoma ($p = 0.03$), and between HSIL and squamous cell carcinoma ($p = 0.03$).

Figure 15 shows median and 25th and 75th percentiles of positive staining cell number, by type of squamous lesion.

In the Figure 16 representative IHC staining of E7 for each cervical lesion are shown.

![Image of Figure 15]

**Figure 15.** Distribution of E7 positive staining cells, by type of squamous lesion (median value: diamond; 25th- and 75th percentiles: lower and upper horizontal lines of the box).
In LSIL and HSIL lesions, the number of cells positive for E7 in the basal, parabasal, intermediate and superficial layer was evaluated, both at nuclear and cytoplasmatic level. The result are showed in Figure 17. Not statistically differences were found between LSILs and HSILs.

Mean cytoplasmatic and nuclear staining intensity in LSIL and HSIL, by layer, is displayed by Figure 18. LSIL and HSIL showed significant difference in the basal ($p = 0.02$), parabasal ($p = 0.009$) and in the superficial layer ($p = 0.007$) at nuclear level.
Results

Figure 17. Mean number of E7 cytoplasmatic and nuclear positive cells, by layer, in LSIL and HSIL.

Figure 18. Mean E7 cytoplasmatic and nuclear staining intensity, by layer, in LSIL and HSIL.
2.3.2. L1

For the evaluation of L1, both cytoplasmatic and nuclear staining was considered. On a total of 138 cervical squamous lesions, we were not able to evaluate 40 due to either non-representative arrayed areas or lost spot during the process of cutting or staining. In the 64 LSILs, the mean number of staining positive cells was 47.5 (SD = 20.0), in the 18 HSILs was 42.2 (SD = 25.3). The 16 squamous cell carcinomas showed a mean number of 16.9 (SD = 19.6). Significant differences were found between LSIL and squamous cell carcinoma ($p < 0.0001$), and between HSIL and squamous cell carcinoma ($p = 0.003$).

Figure 19 shows median and 25th and 75th percentiles of positive staining cell number, by type of squamous lesion.

Figure 20 shows representative IHC staining of L1 for each cervical lesion.
The evaluation of nuclear and cytoplasmic cell positivity by epithelial layer showed that L1 was predominantly cytoplasmatic, both in LSIL and HSIL (Figure 21). In the nucleus, the mean number of positive cells was significantly higher in HSIL than in LSIL in the superficial layer ($p = 0.009$).

Mean cytoplasmatic and nuclear staining intensity in LSIL and HSIL, by layer, is displayed by Figure 22. LSIL and HSIL showed a significant difference in the superficial layer at nuclear level ($p = 0.05$).
Figure 21. Mean number of L1 cytoplasmatic and nuclear positive cells, by layer, in LSIL and HSIL.

Figure 22. Mean L1 cytoplasmatic and nuclear staining intensity, by layer, in LSIL and HSIL.
2.3.3. p16

Both cytoplasmatic and nuclear staining was considered in the evaluation of p16. On a total of 138 cervical squamous lesions, we were not able to evaluate 56 due to either non-representative arrayed areas or lost spot during the process of cutting or staining. In the 50 LSILs the mean number of positive cells were 60.2 (SD = 22.6), in the 18 HSILs was 61.1 (SD = 27.2), among the 14 squamous cell carcinomas the mean number was 37.1 (SD = 28.3). Significant differences were found between LSIL and squamous cell carcinoma ($p = 0.01$), and between HSIL and squamous cell carcinoma ($p = 0.03$).

Figure 23 shows median and 25th and 75th percentiles of positive staining cell number, by type of squamous lesion.

Figure 24 shows representative IHC staining of p16 for each cervical lesion.

Figure 23. Distribution of p16 positive staining cells, by type of squamous lesion (median value: diamond; 25th- and 75th percentiles: lower and upper horizontal lines of the box).
The results of p16 nuclear and cytoplasmatic positivity by layer are shown in Figure 25. No significant differences were found between LSIL and HSIL.

Mean cytoplasmatic and nuclear staining intensity in LSIL and HSIL, by layer, is displayed by Figure 26. LSIL and HSIL showed a significant difference in the parabasal ($p = 0.02$) and in the intermediate layer ($p = 0.01$) at nuclear level.
Figure 25. Mean number of p16 cytoplasmatic and nuclear positive cells, by layers in LSIL and HSIL.

Figure 26. Mean p16 cytoplasmatic and nuclear staining intensity, by layer, in LSIL and HSIL.
2.3.4. hTERT

hTERT was evaluated at both cytoplasmatic and nuclear level. On a total of 138 cervical squamous lesions, we were not able to evaluate 51 due to either non-representative arrayed areas or lost spot during the process of cutting or staining. In the 63 LSILs the mean number of staining positive cells was 47.2 (SD = 28.4), in the 12 HSILs the mean number was 44.2 (SD = 32.3), and in the 12 squamous cell carcinomas was 37.9 (SD = 37.3). No significant differences were found between lesions.

Figure 27 shows median and 25th and 75th percentiles of positive staining cell number, by type of squamous lesion.

Figure 28 shows representative IHC staining of hTERT for each cervical lesion.

![Figure 27](image_url)
The results of hTERT nuclear and cytoplasmatic positivity by layer are shown in Figure 29. A significant difference between LSIL and HSIL was found in the basal layer ($p = 0.01$) at cytoplasmatic level. In the nucleus, the mean number of positive cells in HSIL was higher than in LSIL in all layers ($p < 0.0001$).

Mean cytoplasmatic and nuclear staining intensity in LSIL and HSIL, by layer, is displayed by Figure 30. LSIL and HSIL showed a significant difference in the parabasal layer ($p = 0.04$) at cytoplasmatic level. In the nucleus, the mean staining intensity was significantly different between lesions in the intermediate ($p = 0.02$) and in the superficial layer ($p = 0.02$).
Results

Figure 29. Mean number of hTERT cytoplasmatic and nuclear positive cells, by layer, in LSIL and HSIL.

Figure 30. Mean hTERT cytoplasmatic and nuclear staining intensity, by layer, in LSIL and HSIL.
2.3.5. p53

For the evaluation of p53, only nuclear staining was considered. On a total of 138 cervical squamous lesions, we were not able to evaluate 46 due to either non-representative arrayed areas or lost spot during the process of cutting or staining. In the 65 LSILs the mean number of p53 positive cells were 10.8 (SD = 9.6), in the 13 HSILs was 13.0 (SD = 15.4), in the 14 squamous cell carcinoma the mean number was 11.6 (SD = 17.2). No significant differences were found between lesions.

Figure 31 shows median and 25th and 75th percentiles of positive staining cell number, by type of squamous lesion.

Figure 32 shows representative IHC staining of p53 for each cervical lesion.

Figure 31. Distribution of positive p53 staining cells, by type of squamous lesion (median value: diamond; 25th- and 75th percentiles: lower and upper horizontal lines of the box).
Results

Figure 32. Representative IHC staining of p53 for each cervical lesion (O.M. 40X).

The results of p53 nuclear positivity by layer are shown in Figure 33. A significant difference between LSIL and HSIL was found in the intermediate ($p = 0.02$) and in the superficial layer ($p = 0.03$).

Mean nuclear staining intensity in LSIL and HSIL, by layer, is displayed by Figure 34. LSIL and HSILs showed significant differences in the intermediate ($p = 0.02$) and in the superficial layer ($p = 0.05$).
Results

Figure 33. Mean number of p53 nuclear positive cells, by layer, in LSIL and HSIL.

Figure 34. Mean p53 nuclear staining intensity, by layer, in LSIL and HSIL.
2.3.6. Ubiquitin

For the evaluation of Ub, only nuclear staining was considered. On a total of 138 cervical squamous lesions, we were not able to evaluate 37 due to either non-representative arrayed areas or lost spot during the process of cutting or staining. In the 71 LSILs, the mean number of staining positive cells was 28.1 (SD = 24.6), in the 15 HSILs was 32.7 (SD = 23.4), in the 15 squamous cell carcinomas 53.3 (SD = 30.2). Significant differences were found between LSIL and squamous cell carcinoma ($p = 0.03$).

Figure 35 shows median and 25th and 75th percentiles of positive staining cell number, by type of squamous lesion.

Figure 36 shows representative IHC staining of Ub for each cervical lesion.
Figure 36. Representative IHC staining of Ub for each cervical lesion (O.M. 40X).

The results of Ub nuclear positivity by layer are shown in Figure 37. No significant difference were detected between LSIL and HSIL.

Mean nuclear staining intensity in LSIL and HSIL, by layers, is displayed by Figure 38. LSIL and HSIL showed no significant differences.
Results

Figure 37. Mean number of Ub nuclear positive cells, by layer, in LSIL and HSIL.

Figure 38. Mean Ub nuclear staining intensity, by layer, in LSIL and HSIL.
2.3.7. Ki67

Ki67 is a well known marker used in clinical practice to assess tumour proliferation. For the evaluation of Ki67, only nuclear staining was considered. On a total of 138 cervical squamous lesions, we were not able to evaluate 28 due to either non-representative arrayed areas or lost spot during the process of cutting or staining. As expected, the number of Ki67 positive cells was higher in the high grade cervical lesions. In the 78 LSILs, the mean number of staining positive cells was 17.3 (SD = 11.4), in 18 HSILs was 45.6 (SD = 21.5), in the 14 squamous cell carcinomas was 48.6 (SD = 19.2). Significant differences were found between LSIL and HSIL ($p < 0.0001$), and between LSIL and squamous cell carcinoma ($p < 0.0001$).

Figure 39 shows median and 25th and 75th percentiles of positive staining cell number, by type of squamous lesion.

Figure 40 shows representative IHC staining of Ki67 for each cervical lesion.

![Figure 39. Distribution of Ki67 positive staining cells, by type of squamous lesion (median value: diamond; 25th- and 75th percentiles: lower and upper horizontal lines of the box).]
The results of Ki67 nuclear positivity by layer are shown in Figure 41. Significant differences were detected between LSIL and HSIL in the basal ($p = 0.01$), parabasal ($p = 0.03$), intermediate ($p < 0.0001$) and in the superficial layer ($p < 0.0001$).
2.3.8. Comparison between E7 and the other biomarkers

Repeated measures analysis of variance was performed in order to evaluate differences in the mean number of positive cells between E7 antibody and the other biomarkers. The results are shown in Figure 42 and in Table 11. In all three types of lesion, E7 showed a higher mean value of positive cells than L1, the viral capsid protein. No significant difference was found between E7 and the most used surrogate in clinical setting, i.e. p16, in all types of squamous lesions. In LSIL, the mean number of positive cells stained by hTERT, p53 and Ub, i.e. the most used surrogate in research setting, was lower than E7. Similar differences was found in HSIL where significant difference at 5% level was found between E7 and p53 and Ub. In squamous cell carcinoma E7 and p53 showed a significative difference. E7 and Ki67, a proliferative marker, significantly differed in LSIL and HSIL.
Figure 42. Mean number of positive cells, by marker and type of squamous lesion.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>LSIL</th>
<th>HSIL</th>
<th>Squamous cell ca.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>0.002</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>True surrogate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p16</td>
<td>0.4</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>hTERT</td>
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<td>0.09</td>
<td>1.0</td>
</tr>
<tr>
<td>p53</td>
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<td>&lt;0.0001</td>
<td>0.001</td>
</tr>
<tr>
<td>Ub</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
<td>0.6</td>
</tr>
<tr>
<td>Proliferative marker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki67</td>
<td>&lt;0.0001</td>
<td>0.009</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 11. Comparison between the mean number of E7 positive cells and the mean number of cells stained by six biomarkers, by type of squamous lesion. *p*-values derived from the analysis of variance. In bold type the *p*-values not statistically significant (*p* ≤ 0.05).

Figure 43, Figure 44 and Figure 45 show representative immunostaining of each marker for all the squamous lesions.
Figure 43. Representative IHC of each marker in LSIL (O.M. 40X).
Figure 44. Representative IHC of each marker in HSIL (O.M. 40X).
Squamous Cell Carcinoma

Figure 45. Representative IHC of each marker in squamous cell carcinoma (O.M. 40X).

Spearman’s correlation test and linear regression analyses were performed to investigate the possible correlation between E7 and the other biomarkers. Significant results are shown in Figure 46 and Figure 47 together with scatter diagrams.
Figure 46. Scatter diagrams of E7 versus L1, p16 and hTERT, by type of squamous lesion.
Figure 47. Scatter diagrams of E7 versus p53, Ub and Ki67, by type of squamous lesion.
On the basis of E7 *versus* L1 scatter diagram in LSIL and HSIL, we identified two main groups of lesions. One, the group A, characterized by high level of E7 and low level of L1 (circled in blue in Figure 48) and the group B, characterized by high level of both markers (circled in red in Figure 48). Spots of each lesion were retrieved and re-evaluated by an expert pathologist. In Figure 48 representative images of these two distinct groups are shown. In LSIL, group A is characterized by dysplastic epithelium linked with koilocytosis, while B group is characterized by a condyloma. In HSIL, group A is characterized by high grade dysplasia associated with koilocytosis, while group B lesions display high grade dysplasia without evidence of koilocytotic atypia.
Figure 48. Correlation of mean number of E7 and L1 staining cells in LSIL (left) HSIL (right). In the lower panel, representative images of the two distinct groups, A and B, in LSIL (left) and in HSIL (right) (O.M: 40X).
3. DEEPENING RECQ1 HELICASES FUNCTION (220)

3.1. Expression of RECQ1 in human tumor tissues

The protein expression profile of RECQ1 was analyzed in different types of tumours. A preliminary IHC analysis of perilesional sections of human colon carcinoma, thyroid cancer, lung cancer, and brain glioblastoma tissues showed that RECQ1 was effectively detected in these samples. The RECQ1 expression was confined in the cellular nuclei and more than 30% of cells in each sample positively stained for RECQ1, using either the antibody raised against the full-length protein (RQ-FL) or that one specifically recognizing the C-terminus of RECQ1 (RQ-CT) (Figure 49 A). The specificity of the two anti-RECQ1 antibodies was confirmed by pre-adsorption experiments showing that only a minimal non-specific stain can be detected after pre-adsorption of the tissue with the recombinant RECQ1 protein (Figure 49 B).
Figure 49. (A) Representative IHC against the RQ-FL antibody (left) and the RQ-CT antibody (right) in perilesional tissues from glioblastoma, colon carcinoma, lung cancer and thyroid cancer (O.M 40X). (B) Pre-adsorption experiments by using the recombinant RECQ1 protein on normal colon tissues. Immunostaining against RQ-FL antibody in colon tissues before (1) and after preadsorption with the full-length recombinant RECQ1 protein (2) (O.M. 40X).
Subsequently, the IHC analysis was repeated by including lesional tissues from brain glioblastoma, colon carcinoma, lung and thyroid cancers for comparative analysis (Figure 50). The results showed that RECQ1 was highly expressed both in the perilesional and lesional tissues of these tumors. Only in brain gliomas the expression of RECQ1 was significantly higher in the tumor samples in comparison to the perilesional tissues. In particular, we observed a 4-fold decrease of RECQ1 positive nuclear staining cells from brain tumour tissues versus perilesional ones. These results were also confirmed in autopic normal brain tissues, which might represent a more appropriate control for our experiments since we cannot rule out the possibility that perilesional tissues might be partially influenced in the expression profile by the tumour itself.
Results

Figure 50. (A) Representative pictures of IHC analysis on tissues from different types of tumors. On the left, the perilesional area and, on the right, the tumoral one. IHC was made using the anti-RECQ1 RQ-FL antibody (O.M 40X). The histograms on the right represent the percentage of nuclei positive for RECQ1 in perilesional and lesional tissues of the respective tumors.
Perilesional and autopic normal brain tissues showed a lower number of positive nuclei compared to the other control tissues, suggesting that RECQ1 is less expressed in normal non-dividing tissues (Figure 51). This hypothesis was confirmed by the analysis of RECQ1 in a human normal heart tissue, as another example of non dividing tissue. As reported in Figure 52 no significant nuclear staining for RECQ1 was detected in the heart.

![Figure 51. Representative IHC of RECQ1 expression in normal autopic brain tissues performed using the anti-RECQ1 RQ-FL antibody (O.M. 40X).](image)

![Figure 52. Immunostaining for RECQ1 on human normal heart tissue (O.M 20X).](image)

In order to investigate proliferation activity of glioblastoma tumours, IHC staining of Ki67 was performed. As it is known, the protein is present during all active phases of the cell cycle (G₁, S,
G₂, and mitosis), but it is absent in resting cells (G₀). This is the reason why Ki67 is routinely used in clinical practice for determining the so-called growth fraction of a given cell population. The proliferation index (P.I.), *i.e.* the percentage of nuclei staining positively for this marker, increases as the growth fraction rises (221).

Double IHC staining for Ki67 and RECQ1 (Figure 53) demonstrated that proteins frequently co-localize, but RECQ1 is expressed also in non-replicating cells. This observation was more evident in normal colon tissue, which has a higher replicative status. Colon tissues IHC showed that Ki67 was expressed in the crypts, indicating their proliferation state, while RECQ1 was also present throughout the apical cell surface (Figure 54).

![Figure 53](image-url)

*Figure 53. Representative double immunostaining against Ki67 (in red) and RECQ1 (in brown) in multiform glioblastoma (A) and colon tissues (B) (O. M. 40X). Arrows indicate cells in which the two proteins co-localize.*
In summary, the IHC analysis of different tumour samples indicates that RECQ1 is highly expressed in all types of tumours. However, only in the case of glioblastoma a higher expression of RECQ1 was significantly associated to neplastic transformation because of the lower expression of RECQ1 in brain peri-lesional tissues.

Figure 54. Representative immunostaining against Ki67 (in red, A) and RECQ1 (in brown, B) in normal colon tissues (O.M. 20X).
3.2. Tissue microarray analysis

To further validate the results on RECQ1 expression in glioblastoma, its expression pattern was analyzed on a tissue microarray containing a total of 63 glioblastoma and 19 perilesional tissues (Figure 55).

In line with the previous results, the percentage of RECQ1 positive cells (Figure 56) and the intensity of the staining (Figure 57) were significantly higher in tumoral versus perilesional tissues ($\rho = 0.001$ and $\rho = 0.0009$, respectively). The higher positivity of glioblastoma to RECQ1 correlated with higher intensity of the immunostaining (Spearman’s $\rho = 0.5$, $\rho = 0.0001$). In particular, RECQ1 positive cells in lesional tissues displayed strong nuclear positivity with a staining nuclear intensity of 3+, while most of the positive cells for RECQ1 in the perilesional tissues were characterized by a weaker staining intensity of 2+. The number of positive cells for Ki67 differed significantly between tumours and matched surrounding normal tissues, with a higher expression of the protein in the lesional tissues ($\rho = 0.0008$) (Figure 58).
Results

Figure 56. Distribution of cells positive to RECQ1 in 19 brain glioblastomas and paired peri-lesional tissues. The boxes summarize the confidence interval (lower and higher horizontal lines), 25th-50th and 75th percentiles (horizontal lines of the box).

Figure 57. Distribution of the staining intensity (2+ in light gray, 3+ in dark gray) for RECQ1 in 19 brain glioblastomas (left) and paired peri-lesional tissues (right).
Figure 58. Distribution of cells positive to Ki67 in 19 brain glioblastomas and paired peri-lesional tissues. The boxes summarize the confidence interval (lower and higher horizontal lines), 25th-50th and 75th percentiles (horizontal lines of the box).

GFAP staining showed that in the peri-lesional area astrocytes made up the vast majority of all positive cell for RECQ1 while a lesser positive staining for RECQ1 was detected in the oligodendrocytes and the support neurons (Figure 59). GFAP staining highlighted that only astrocytes presented an intense nuclear positivity to RECQ1 in tumour tissues.
Figure 59. Representative GFAP staining in a lesional (left) and peri-lesional (right) glioma tissue. Arrows indicate the astrocytes (A), which are positive to the antibody, the oligodentrocytes (O) and the support neurons (N) (O.M. 40X).

A detailed IHC analysis of the glioblastoma in the TMA indicated that the percentage of positive cells for Ki67 and RECQ1 did not differ significantly between males and females ($p = 0.9$ and 0.7, respectively). Similarly, the age at diagnosis did not correlate with the percentage of positive cells for RECQ1 and Ki67 ($p = 0.4$ and $p =1.0$), and the intensity of signal for RECQ1 ($p = 0.5$). A significant association was detected between the percentage of positive cells and the intensity of the signal for RECQ1 (Spearman’s rho = 0.5, $p = 0.0001$) indicating that samples with a high number of positive cells are associated with a stronger signal. No correlation was present between the percentage of positive cells for RECQ1 and Ki67 (Spearman’s rho = 0.2, $p = 0.2$). In summary, the TMA analysis confirmed that RECQ1 expression was significantly increased in the tissues surrounding glioblastoma.
3.3. Analyses of RECQ1 down-regulated cells

Protein expression analyses of RECQ1 on glioblastoma tissues suggest that the helicase is almost absent in perilesional and/or normal tissues, possibly because of the low degree of proliferation of brain cells. Hence, we believe that RECQ1 could be a potential target for chemotherapy in brain tumours, since its depletion by RNAi or its inhibition by selective compounds could involve only neoplastic cells. For this reason, we tested this hypothesis in two different glioblastoma cell lines (T98G and U87), silencing the RECQ1 gene and monitoring the effects at protein level by means of Western blotting, immunofluorescence and FACS analysis. Normal human IMR-90 fibroblasts, transiently transfected with RECQ1-specific siRNAs and cells transfected with a luciferase siRNA duplex were used as control. Western blot analysis showed that in T98G and U87 there was more than 80% depletion of RECQ1 in all whole cell extracts upon the silencing of RECQ1-gene as compared to control cells (Figure 60).

![Western blot analysis of T98G, U-87 and IMR-90 cell lines transiently transfected with a pool of siRNA against RECQ1. α-Tubulin was used as loading control. Abbreviation: L, Luciferase silencing; R, RECQ1 silencing.](image)

Figure 60. Western blot analysis of T98G, U-87 and IMR-90 cell lines transiently transfected with a pool of siRNA against RECQ1. α-Tubulin was used as loading control. Abbreviation: L, Luciferase silencing; R, RECQ1 silencing.
3.4. Cell cycle analyses

Previous studies showed that siRNA-mediated depletion of RECQ1 impaired cellular proliferation in different cell lines (205). This observation was also supported by FACS analysis of RECQ1-depleted T98G glioblastoma cells that had been bromodeoxyuridine (BrdU)-labelled, indicating that there is more than 50% reduction in both BrdU labelling and S phase fraction. This reduction of S phase cells is associated to an increased fraction of cells arrested in G1 (Figure 61). These results confirm that RECQ1 depletion suppresses cell proliferation by interfering with DNA synthesis.

Figure 61. (A) Flow cytometry profiles of DNA content (x-axis; propidium iodide/PI staining) versus BrdU incorporation (y-axis; anti-BrdU immunostaining) 72 h after siRNA transfection. Boxes are labeled to indicate cell cycle phases. (B) The bar graph at bottom reports the percentage of G0/G1, S-phase/BrdU positive and G2/M cells in cultures that had been transfected with RECQ1 (siRNA RECQ1) or luciferase/control (siRNA Luc) siRNA pools. Results shown are the mean ± SE from three independent experiments.
3.5. Colony forming assay

To test the role of RECQ1 in glioblastoma cell growth and proliferation, we compared the colony forming properties of the T98G and U-87 glioblastoma cell lines and normal human IMR-90 fibroblasts, after the transient transfection with RECQ1-specific siRNAs, versus cells transfected with a luciferase siRNA duplex as control. The colony forming assays demonstrated a significant reduction in both size and number of colonies of RECQ1 downregulated T98G and U-87 glioblastoma cells (Figure 62). On the other hand, the downregulation of RECQ1 in normal human primary fibroblasts did not significantly affect the proliferation capacity of these cells, in agreement with previous findings (205). In particular, RECQ1-depleted glioblastoma cells showed about 10-fold reduction in their proliferative capacity in comparison with control cells (from 5% to 59%). Collectively, this data point to a specific regulatory role of human RECQ1 in the proliferation in glioblastoma cells.
Figure 62. (A) Clonogenic assays performed in RECQ1-depleted T98G, U-87, and IMR-90 cell lines. Pictures show colonies formed after seeding 800 cells. (B) Bar-graphs showing the plating efficiencies expressed as colony forming capacity. Values represent the average ratio of the number of formed colonies to the number of cells seeded, expressed as percentage.

Moreover, in order to provide additional data on the role of RECQ1 in DNA synthesis and cell proliferation, the colony forming capacity of RECQ1-depleted cells upon replication stress induction with HU was measured. Cellular survival curves at increasing HU concentrations showed that RECQ1 depleted cells were hyper-sensitive to HU treatment, suggesting a possible role of RECQ1 in DNA replication fork processing (Figure 63).
To explore the possibility that RECQ1 might represent a suitable new target for brain tumour treatment, we investigated the sensitivity of glioblastoma cells to temozolamide (TMZ), a commonly used anticancer agent in the treatment of human brain tumours (222, 223). TMZ is an alkylating agent that effectively inhibits glioblastoma cell proliferation. Its activity is primarily related to the formation of O6-methylguanine in DNA, which mispairs with thymine during DNA replication cycles and leads to cell death because of accumulation of unrepaired DNA mismatches. (224, 225). Thus, the colony forming capacity of RECQ1-depleted (205) glioblastoma cell lines after treatment with TMZ was analyzed (Figure 64). Cellular survival curves related to increasing TMZ concentrations showed that RECQ1 depleted T98G and U87 cell lines were hypersensitive to the action of TMZ suggesting a possible role of RECQ1 in DNA repair pathways linked to DNA replication. These data also suggest that RECQ1 might represent a suitable new target for the inhibition of cell proliferation in brain tumours.
Results

Figure 64. The graphs show the cellular surviving fractions measured at different doses of temozolomide in control and RECQ1-depleted T98G and U-87 cells. Surviving fraction values are the mean ± SEM from three independent experiments.

3.6. Immunofluorescence analyses

The reduced cell proliferation and the cell cycle perturbation taking place upon RECQ1 down-regulation might be related to some type of chromosomal insult or damage occurring in the RECQ1 down-regulated cells leading to replication arrest and DNA surveillance machinery activation. Thus, the rate of foci formation of two DNA damage/repair markers, γ-H2AX and RAD51, was analyzed by immunofluorescence.
3.6.1. γ-H2AX foci formation

An early response to DNA damage is the phosphorilation of H2AX, a variant of Histone H2A, at the level of Serine 139. The analysis of γ-H2AX foci provides a quantitative procedure to monitor DNA breaks (226). Previous studies with RECQ1-deficient HeLa cells showed an increased level of DNA damage and sister chromatid exchanges upon RECQ1 depletion (227). Immunofluorescence experiments on glioblastoma cells indicated that RECQ1 depletion results in a dramatic increase in spontaneous γ-H2AX foci formation confirming that the reduced expression of RECQ1 is associated with defects in DNA repair (Figure 65). Approximately 50% of the RECQ1 depleted cells contained more than 10 γ-H2AX foci per nucleus, compared to only 15% of the wild-type cells. Western blots against γ-H2AX confirmed an increased load of DNA lesions upon RECQ1 depletion (Figure 66).
Results

Figure 65. (A) Representative immunofluorescence staining of endogenous RECQ1 and endogenous γ-H2AX on T98G cells after treatment with anti-RECQ1 siRNA or control siRNAs (anti-Luciferase). (B) The bar-graph shows the percentage of cells that contain a defined number of γ-H2AX foci per cell.
3.6.2. RAD51 foci formation

RAD51 is a key component of the machinery and a marker for activation of homologous recombination (HR) repair pathway upon DNA double strand break induction. Previous studies suggested that RecQ helicases play an important role in HR repair at sites of chromosomal DNA damage (192). To test if RECQ1 is also involved in this pathway, we analyzed the ability of RAD51 to form foci in RECQ1 depleted T98G cells. As shown in Figure 67 the RECQ1 glioblastoma depleted cells exhibited an increased number of spontaneous RAD51 foci relative to the control cells. However, the increased number of RAD51 foci is lower than number of the γ-H2AX foci (Figure 67). This observation suggests that the double-strand breaks might not be the major form of damage that leads to γ-H2AX foci formation in the absence of RECQ1.
Figure 67. (A) Representative immunofluorescence staining of endogenous RAD51 and BrdU incorporation on T98G cells after treatment with anti-RECQ1 siRNA or control siRNAs (siRNA Luc). (B) Bar-graph showing the percentage of cells that contain a defined number of RAD51 foci per cell.

Figure 68. Western blot analysis of T98G and U-87 cell lines after transient depletion of RECQ1 gene. α-Tubulin was used as loading control. Abbreviation: L, Luciferase silencing; R, RECQ1 silencing.
DISCUSSION

Thanks to the possibility of having accessing to the fresh and archival samples from several hospitals, our laboratory can investigate the potential of molecular technological innovations directly on the clinical material and exploit them on clinical case studies. My PhD project aimed at:

1) The standardization of methods devoted to a reliable protein extraction and analysis from FPE tissues;

2) The application of protein analysis in two different research settings:

   (a) at diagnostic level: the validation of E7 HPV antibodies in cervical cancer.

   (b) at translational research level: the identification of a new therapy target for glioblastoma, deepening the functions of human RECQ1 helicase.

1. STANDARDIZATION OF PROTEIN ANALYSIS OF TISSUES FIXED WITH FORMALIN AND FINEFIX (132)

Up to now, the lack of standardized guidelines for the DNA, RNA and protein analysis of AT have hampered the use of such samples for molecular analyses. In 2007, the European project IMPACTS was established with the main goal of standardizing molecular methods in AT for the first time. My research, developed within the IMPACTS work-package on proteomics, contributed to: (a) new fixation procedures compatible with subsequent protein analysis; (b) novel protein extraction technologies in FPE samples.

In this study, a total of 81 lysates were examined. As Western blot analysis demonstrated, all the 5 laboratories involved in the project were able to extract proteins from both formalin and FineFix
tissues. All the lysates obtained were found to be reliable for protein microarray application. Our quantitative comparison between fixatives for tested antibody panel (β-Actin, E-cadherin, EGFR, HER2, and ER) yielded a higher immunostaining intensity for the FineFix lysates compared to the FFPE ($p = 0.04$). Remarkable differences for two large membrane proteins, EGFR and HER2 were found. Signal intensities for EGFR ($p =0.007$) and HER2 ($p =0.02$) were significantly higher in the FineFix samples compared to the FFPE samples, whereas no differences were observed for β-Actin, E-cadherin, and ER. A recent study by Nassiri et al. showed a similar discrepancy for HER2 abundance between matched UMFIX/FFPE breast cancer samples, whereas they were not able to find differences for the ER (228). The reason why EGFR and HER2 gave stronger signals in the FineFix samples could be that alcoholic formalin-substitutes, like FineFix and UMFIX, improve protein detection because of their non-crosslinking property. Most likely, in FineFix samples, proteins could be extracted out the tissue context more easily. Based on our results, we conclude that fixation treatment could affect protein analysis, since in the FineFix lysates the immunoreactivity was higher than in formalin ones. Furthermore, our study confirms those previously reported (91, 92) which demonstrated the application of quantitative protein analysis is feasible in FFPE tissue.

Overall, our study further demonstrates that FineFix may be used as a formalin-substitute for protein analysis, because IHC staining and morphology on FineFix tissues are comparable to those of formalin-fixed tissues, the gold standard for pathological evaluation (131).

2. HPV MoAbs VALIDATION

The detection and typing of HPV in pathology specimens is gaining increasingly in importance, but to date only surrogate biomarkers are available to detect HPV infection at the protein level. This research was carried out in collaboration with a private company with the following two main goals:
1. To evaluate a new HPV specific antibody in comparison to other markers used in clinical practice and research setting.

2. To evaluate the usefulness of the new HPV antibody for the morphological diagnosis of cervix uteri lesions.

The gold standard for the diagnosis of pre-malignant cervical lesions is the identification of well-described morphological features in biopsies stained with H&E (229). Nevertheless, the histological diagnosis of precancerous lesions, like CIN, with H&E is subject by a significant degree of discordance among pathologists (230-233). The use of adjunctive IHC stain can help increase the accuracy of the diagnostic interpretation of cervical lesions and reduce false-negative and false-positive diagnosis. Many IHC studies (182, 186, 234) have provided evidence that Ki67 and p16 are valuable surrogate markers and can improve the diagnosis of difficult interpretation of cervical biopsies. The analyses of an antibody directed against the viral oncoprotein E7 were performed for the first time by the present study. The evaluation of the staining efficiency was done by comparing IHC results of the new antibody with those obtained with the surrogate markers p16, hTERT, p53 and Ub, with Ki67 (marker of cell proliferation) and with the HPV capsid protein L1.

2.1. E7 IHC

The evaluation of E7 was preceded by the screening of 39 MoAbs directed against the E7 viral oncoprotein (see the Results paragraph 2.1, pag. 53 and the summary Table 9, pag 28) The screening allowed me to obtain two important pieces of information: (a) the usefulness of the different MoAbs in differential diagnosis because of the different specificities of these molecules; (b) the selection of a proper antibody for further validation analyses and comparison with the other markers.
The specificity of this new antibody for the E7 oncoviral protein was confirmed by the preadsorption test and the western blot analyses (Figure 13 and Figure 14, pag. 56).

The IHC analysis of the number of positive cells (Figure 15, pag. 59) showed significant differences between LSIL and squamous cell carcinoma ($p = 0.03$), and between HSIL and squamous cell carcinoma ($p = 0.03$). This findings could suggest that the viral protein is mainly responsible for the initiation and promotion of the infection rather than for its progression.

The analyses conducted in the epithelial tissues, layer by layer, showed that E7 stained both nucleus and cytoplasm in all the 4 layers of the LSIL and HSIL (Figure 17, pag. 61). The cytoplasmatic positivity is higher than in the nuclear one in all layers and in both types of lesion, even if no significant differences were found. The fact that E7 showed both nuclear and cytoplasmatic positivity indicates that the protein could have different post-translational modifications and functions probably affecting its sub-cellular localization.

### 2.2. p16 and Ki67 IHC

p16, together with the evaluation of Ki67, is considered a valuable surrogate marker of HPV infection in cervical lesions (167, 181, 183, 184, 186, 235). All these studies reported that p16 expression increases with the severity of the lesions, by considering as outcome measure the staining intensity score and the distribution of the staining through the epithelium thickness. Generally, positive immunostaining for both p16 and Ki67 in the upper two thirds of the squamous epithelium is a good indicator of atypical squamous lesions (236). Our results on the staining intensity (Figure 26, pag. 67) showed that in the nucleus, the HSIL displayed a higher (237) staining intensity in each layer in comparison to LSIL. The difference was significant in the parabasal (mean intensity 2.3 vs 1.7; $p = 0.02$) and in the intermediate layer (2.2 vs 1.5; $p = 0.01$). On the other hand, no significant difference was found in cytoplasm.
In this study the number of cells stained positive for p16 was lower in squamous cell carcinoma than in LSIL and HSIL. This could be due to the immortalization and uncontrolled proliferation of cancer cells. The p16 protein works as an negative regulators of the cell cycle progression through the inhibition of cyclin-dependent kinases 4 and 6 and interactions with cyclin D1. In response to infection by high risk HPV, p16 is overexpressed leading to a cell cycle arrest (167). During cancer progression, the cell cycle control is lost and cells become immortalize. Moreover, I have to consider that hypermethylation of p16 gene, frequently observed in cervical cancer (238-244), that could lead to the inactivation of the gene transcription by suppressing the control of the p16 protein on cell cycle progression.

Many authors recommend the use of p16 IHC in diagnostic because p16 staining can facilitate the interpretation of equivocal biopsies. On the other hand, other authors indicate that p16 shows a higher specificity than sensitivity for squamous lesions which reduces its usefulness in diagnostics (183). For this reason, it was recommended the use of a combination of immune-staining, with p16, L1 and Ki67, to better classify cervical lesions, especially those that might progress (182, 245, 246).

My results concerning Ki67 IHC (Figure 39, pag. 77), which is a well known cell proliferation marker, confirm that its expression increases with the severity of cervical lesions, as already reported (186, 235).

### 2.3. hTERT, Ub and p53 IHC

Many other dysplasia-associated biomarkers have been identified and used to improve the diagnostic accuracy of pre-neoplastic and neoplastic lesions, such as hTERT, Ub and p53 (187, 189, 234). During the infection, E6 and E7 HPV oncoproteins activate different molecular mechanisms leading to malignant transformation. These mechanisms are mediated by proteins which are used as molecular markers and indicators of HPV infection.
2.3.1. hTERT IHC

Multiple studies have analyzed telomerase as possible marker for HSIL and invasive cervical cancer (187-189, 247-249). The E6 oncoviral protein activates the oncogenic hTERT enzyme, by stimulating the transcription of the gene and by immortalizing transformed cervical cells. Reported studies have shown that the activity of this enzyme, measured by the TRAP (Telomeric Repeat Amplification Protocol) method, increases in cervical cancer in comparison to normal cervical tissue (250, 251). However, my results showed that the mean number of positive cells for hTERT was higher in the LSIL and in the HISL in comparison to squamous cell carcinoma (Figure 27, pag. 68). The discrepancy between our results and those indicating higher telomerase activity in cancer, could be explained by the methodological differences. TRAP is a measure of telomerase activity, while IHC measures the protein presence, and its accumulation. Telomorase is active only if it is assembled with other elements in a functional complex. Nevertheless, the existence of alternative spliced forms of hTERT mRNA or by the post-translational modification of hTERT protein could also explain our results. Considering nuclear and cytoplasmatic positivity by layer, cytoplasmatic hTERT expression was found both in LSIL and HSIL, whereas nuclear positivity is virtually absent in the LSIL. The mean number of nuclear positive cells in HSIL was higher than in LSIL in all the epithelial layers ($p < 0.0001$). In line with the results reported by Kyo et al. (252), my observations demonstrated that hTERT is expressed not only in nucleus but also in cytoplasm of cancer cells. Further studies on the different localization of hTERT are needed in order to investigate if this staining pattern is associated with pathological features of the lesions or with their progression.

2.3.2. Ub and p53 IHC

My results (Figure 35, pag. 74) showed a statistical difference ($p = 0.03$) in the mean number positive cells for Ub between LSIL and squamous cell carcinoma. As far as p53, (Figure 31, pag. 71), even if no statistical differences were found, the median number of positive cells was lower in squamous cell carcinoma (median = 4) than in LSIL and HSIL (median = 10). The rapid removal of
enzymes and proteins, generally by means of the ubiquitin-proteasome pathway, is essential for the control of cellular growth and metabolism. In the process of cervical carcinogenesis, the binding of the viral oncoproteins with ubiquitin and other related proteins modified and induced degradation of different proteins, such as p53 (Figure 4 and Figure 5, pag. 21). Thus, not only are cell proteins functionally inactive, but their expression levels tend to be lower in relation to the intense degradation. As a consequence, with the increase of lesion severity, the IHC expression of Ub in neoplastic cells was higher, whereas that of p53 is lower (Figure 35, pag 74 and Figure 31, pag. 71).

2.4. L1 IHC

L1 is a viral capsid protein and is expressed with the production of infectious viral particles. Transcription of this late protein seems to be regulated by cell-derived transcriptional factors that are produced by the differentiated cells of the intermediate and superficial layer. My results, in agreement with previous studies (182, 230, 245), showed an higher positivity of L1 protein in LSIL than in HSIL and in squamous cell carcinoma, indicating a viral productive phase of infection (Figure 19, pag. 62). A statistically significant differences were detected between LSIL and squamous cell carcinoma and between HSIL and squamous cell carcinoma ($p < 0.0001$ and $p = 0.003$, respectively). However, unlike what reported by previous studies which found L1 mainly expressed in the nucleus, I found L1 mainly expressed in the cytoplasm of LSIL and HSIL and a positive nuclear staining predominantly observed only in the superficial layer of HSIL.

2.5. Comparison of E7 with the other markers

Different studies consider the combination of HPV L1, p16 and Ki67 immunostaining as valuable index for diagnosis and prognosis of cervical lesions (182, 245, 246), because up to now there were no available antibodies directed against E7 oncoprotein.
The new HPV antibody, directed against the E7 oncoviral protein, gave me the opportunity to compare this antibody with the viral capsid protein L1, with the surrogates markers p16, p53, Ub and hTERT, and with the proliferative marker Ki67 for the first time ever. In my analyses I used the number of positive stained cells for each marker as measure of outcome because it is a less subjective method of evaluation. The results (Figure 42 and Table 11, pag. 80) showed that the E7 immunostaining is similar to that of p16, which is the most used surrogate marker in clinical practice for all types of squamous lesions. Moreover, in the HSIL the IHC results of E7 are similar to that of hTERT. In the detection of squamous cell carcinoma, E7 is similar to hTERT and Ub. At the histological examination, the staining pattern in each epithelial layer (Figure 43, pag. 81, Figure 43, pag. 82 and Figure 45, pag. 83) was very similar between E7 and these surrogates. So we can conclude that the new E7 antibody is a suitable non-surrogate marker for the diagnosis of cervical lesions.

Comparison between the mean number of E7 and L1 positive cells shows a significant difference in LSIL ($p = 0.002$), in HSIL ($p = 0.01$) and in squamous cell carcinoma ($p=0.01$) (Table 11, pag. 80). Moreover, the ratio of the means between E7 and L1 positive cells indicate that in LSIL, E7 is 1.2 times higher than L1 (58/47), whereas, in HSIL and in squamous cell carcinoma this value increases to 1.5 (63/42) and 2.5 (63/16), respectively. L1 capsid protein is mainly detectable during the productive stage of HPV disease. A high expression level of L1 indicates that the virus is present as a productive form. On the other hand, a low expression level of L1 could indicate a latent viral infection or integration of HPV DNA into the host genome. Similarly, the expression level of E7 is related to the degree of dysplasia: the up-regulation of the oncoprotein is mainly associated with the integration of the viral DNA into the host genome. In agreement with the molecular biology of the infection, my results indicate that in LSIL there is an early dysplastic state associated with the production of the virion. As the lesions increase in severity, the ratio of the means increases, indicating that the advanced dysplastic features are less associated with virions assembly. Correlation analysis between these two viral proteins allows to identify two possible
groups of lesions, both in LSIL and HSIL (Figure 48, pag 87). This observation was supported by microscopical analyses. The review of the slides by expert pathologists leads us to suggest two possible models in the progression of LSIL: the replicative and the proliferative (Figure 69). The replicative model (Figure 69 A) is characterized by the nuclear and cytoplasmatic positivity of the two proteins, which increases from the basal to the superficial layer. In the replicative model we postulated that morphological findings are due to an active viral replication of the HPV. We hypothesize that this model could be associated to a low or intermediate risk of progression of the disease. On the other hand, the proliferative model (Figure 69 B) shows a basal layer characterized by a higher number of positive cells (both nuclear and cytoplasmatic) than the superficial layer. We hypothesize that this model could be mostly related to cell proliferation, and that this staining pattern could be associated with a high risk of progression of the lesion. The results of this study show that the combination of the new E7 antibody and the L1 staining may allow a distinction help differentiate between varying risk patterns for low grade lesions and could have possible applications also in cytology. Further analyses are needed to validate these hypotheses, based on specific clinical case studies, aiming at improving the quality of diagnosis, gaining more information about the risk of progression and facilitating the therapy decision.
IHC and ICC are the most widely used diagnostic tools in the clinical practice requiring simpler handling and management procedures compare to PCR or hybridization methods (e.g. Southern blot). Although useful as screening tools, HPV DNA tests have some technical limitations. This type of analysis displays high sensitivity (>90%) for detecting precancerous lesions, but it is unable to differentiate among latent, subclinical and clinical relevant infections, which are important for the early diagnosis and risk assessment. Furthermore, PCR-based test need more specific laboratory equipments and tools compare to IHC which is a “mature technology”, widely used in every hospitals of the world. Currently, IHC and ICC techniques are not routinely used for the diagnosis of HPV infections because there are no specific biomarkers and the interpretation of results is often
characterized by a poor inter- and intra-observers agreement. As my research demonstrated, the availability of this new specific antibody against the E7 viral protein, can help the morphological evaluation of cervical lesions linked to HPV infection and the potential evolution of risk progression through a commonly used diagnostic methodology.

3. DEEPENING THE RECV1 HELICASE FUNCTION

The RECQ protein family is a highly conserved group of DNA helicases with diverse roles in multiple DNA metabolic processes, including DNA recombination, replication and repair, and a possible role in transcription. Genetic defects in three of the five human RECQ helicases, BLM, WRN and RECQ4 can lead to high levels of genomic instability and, in humans, to premature aging and increased susceptibility to cancer. These phenotypes reflect the prominent role that human RecQ helicases have in maintaining genome stability and in the response to cellular stress and/or DNA damage. Mutations in the RECV1 and RECQ5 genes may be responsible for additional cancer predisposition disorders, but this remains to be proven. Allelic losses or deletion of chromosome 12p12, where the RECV1 gene is located, is a frequent event in a wide range of solid tumours (253-256), and a single-nucleotide polymorphism of the RECV1 gene has been associated with a reduced survival of pancreatic cancer patients (204, 257). In this regard, RECQ helicases might be considered as “tumour suppressors” that prevent neoplastic transformation by controlling chromosomal stability. However, recent studies reported that the BLM and WRN are up-regulated in tumours. Hickson et al. showed that BLM is highly expressed in tumour cells of both lymphoid and epithelial origin and that this reflects the greater fraction of proliferating cells that are present in tumours relative to the normal tissues of the same origin (201). Similarly, Kobbe et al. suggested that WRN could also be involved in the promotion of tumour cell growth (202). A cancer specific role of RECV1 is supported by two recent reports showing that RECV1 silencing in cancer cells resulted in mitotic catastrophe and local and systemic administration of RecQL1-siRNA mixed with polyethyleneimine polymer or cationic liposomes prevented tumour growth in murine models (205, 206).
In order to better understand the function of RECQ1 in tumours, during my doctorate I first characterized the protein profile of RECQ1 in tumours of different origin by IHC analyses (Figure 50, pag. 91). Previous results suggested that RECQ1 is highly expressed in all tumours analyzed, in agreement with previous findings (258, 259). Nevertheless, only in glioblastoma a higher expression of RECQ1 was significantly associated to cancer because of the lower expression of RECQ1 in perilesional tissues ($p = 0.0013$). Moreover, my results showed that RECQ1 expression is confined in the nuclei of the tumour cells, suggesting that this protein plays an important role in glioblastoma growth. This result was validated on a TMA containing a total of 63 glioblastoma and 19 perilesional tissues (Figure 55 and Figure 56, pag. 95-96); this analyses indicated also that the higher expression of RECQ1 in the tumours is not related to individuals’ sex or age. As expected, IHC analyses against Ki67 showed that protein expression is higher in the glioblastoma tumours than in the surrounding perilesional tissue ($p = 0.0008$) (Figure 58, pag. 97). This results further confirmed that the lower expression of RECQ1 in perilesional glioblastoma cell is possibly due to the low degree of proliferation of brain cells. Hence, we believe that RECQ1 would be an ideal target for chemotherapy especially in the case of brain tumours since its depletion by RNAi or its inhibition by selective compounds would primarily affect tumoural cells (260). However, double IHC staining of RECQ1 and Ki67 (Figure 53, pag. 93) indicated that the two proteins frequently co-localize, but RECQ1 helicase expression is not exclusively related to the cell replication stage. My results did not show any correlation between the percentage of positive cells for RECQ1 and Ki67 (Spearman’s rho= 0.2, $p = 0.2$), suggesting that the high expression of RECQ1 in brain tumours is not simply related to the higher degree of proliferation of these cells.

To further investigate the function of this human helicase in glioblastoma, different cell lines experiments were made. An essential role of RECQ1 in tumour growth and proliferation was confirmed by the clonogenic assays on T98G and U-87 cells. After RECQ1 gene silencing, a significant reduction in the number of colonies was observed (Figure 62, pag. 102). This event is supported by a previous study showing that RECQ1 is important for HeLa cell proliferation and plays a unique role in the maintenance of genome integrity (227, 261). Consistently, I found that
Discussion

the silencing of RECQ1 results in spontaneous γ-H2AX foci formation and HU hypersensitivity in T98G cells (Figure 66, pag. 107, and Figure 63, pag. 103), thus suggesting that RECQ1 plays an important and unique role in DNA repair during DNA replication. Concerning the specific role of RECQ1 in genome maintenance Sharma et al. suggested that RECQ1 might be involved in the regulation of the homologous recombination pathway of DNA double-strand break repair (227). Interestingly, immunofluorescence experiments indicate that RECQ1 loss resulted in fewer RAD51 foci formation in comparison to γ-H2AX foci (Figure 67, pag. 108). This observation is indicative that RECQ1 might not play a major role in homologous recombination (HR). Moreover, additional results showed that there isn’t any significant defect in HR frequency in RECQ1 siRNA-inhibited cells. The hyper-recombination phenotype of RECQ1-depleted cells suggests two hypothesis: (a) RECQ1 could be involved in the suppression of some illegitimate recombination events, as already proposed for other helicases of the same family (262); (b) loss of RECQ1 could result in the accumulation of some form of DNA lesion or strand breaks, other than DSBs, that might subsequently lead to repair by HR if not properly repaired.

The resistance of glioma cells to TMZ is mainly associated with levels of DNA repair protein O6-alkylguanine alkyltransferase (AGT) which removes alkyl groups at O6 position of guanine. O6-benzylguanine (O6-BG), an inhibitor for AGT, reduces resistance to TMZ (263). It has been already demonstrated that chemosensitivity of tumour cells to TMZ correlates with the inhibition of telomerase activity (264). Our studies using malignant glioma cell lines with low (U87-MG) and high levels of AGT (T98G) showed that RECQ1 suppression by RNA interference increases the sensitivity of these cells to TMZ, independently of the AGT expression levels (Figure 64, pag. 104). These findings support the notion that RECQ1 plays a unique role in DNA repair during DNA replication in malignant cells.

A recent study showed that RECQ1 silencing in cancer cells induces a cell specific mitotic catastrophe not observed in normal cells (205). The absence of RECQ1 might promote the accumulation of DNA damage in the M-phase arrested cells due to deficient G1 and G2 checkpoint
functions of cancer cells. These events would then lead to the specific mitotic death of this cell type. Under normal conditions, the accumulation of DNA damage and mitotic cell death is avoided during S and G2 phases by the up-regulation of different repair enzymes, such as RECQ1. Thus, cancer cells might maintain a greater copy number of DNA repair enzymes to restore DNA damage in a short time. My results demonstrated that, similarly to other RecQ helicases, RECQ1 plays an important role in the regulation of cellular proliferation by interfering with DNA synthesis. Cell cycle analyses of RECQ1-depleted T98G glioblastoma cells indicated that there is a reduction of S phase cells associated to an increased fraction of cells arrested in G1 (Figure 61, pag. 100).

The fact that RECQ helicases, such as BLM, WRN and RECQ1, are up-regulated in tumours might appear incompatible with their suggested tumour suppression function. A possible explanation is that, in somatic cells, DNA repair defects affecting genome integrity due to a RECQ helicase deficiency may lead to cancer predisposition. Conversely, increased RECQ helicase expression might be required in transformed or actively proliferating cells to resolve and repair the greater load of DNA intermediates that are generated during active replication.

Taken together, our results indicate that RECQ1 might be considered as a new suitable target for the development of anti-cancer therapies to eliminate proliferating tumour cells (260).
CONCLUSION

Microscopical examination of neoplastic lesions has been the starting point for diagnosis, prognosis and therapeutic decision. However, patients with morphologically identical tumours can have different clinical outcomes. To address the pressing medical need for more accurate predictions, a variety of technologies (DNA microarrays, quantitative RT-PCR, RPPA) have been developed over the last four decades to sub-classify cancers on the basis of their molecular features. Translational research aims at translating molecular tools in clinical practice to better define diseases with same morphological features.

Conventional diagnostic and therapeutic approaches for human cancers are currently based on a combination of histopathology and immunohistology, integrated with clinical data. In most cases, this strategy provides precise information on tumour primary site, histological type, stage and grade as well as information on the completeness of surgical removal. A profound impact on diagnostic pathology is coming from the recent advances in molecular biology and molecular technologies which are increasing their potential in complementing the traditional histopathological analyses. In particular, molecular information derived from genomics, transcriptomics and proteomics will be of critical importance to deeper understand molecular pathogenesis, to increase treatment efficacy, and to enhance the quality of information provided to clinicians in order to improve patient management (265).

Worldwide, the most abundant supply of human tissues is the FFPE blocks. In fact, all tissues taken for any clinical reason are fixed and paraffin-embedded and, after the definitive histological diagnosis, are stored in the pathology archives sometimes for decades or more. These archives represent a vast, well-characterized historical collection covering virtually every disease with long follow-up periods, a prerequisite for optimal retrospective studies (241). It has been estimated that in the last twenty years more than billion FFPE patients’ specimens were stored in European hospitals. In the last decades, the FFPE blocks have been widely used for molecular analyses on
DNA, RNA and, more recently, proteins, thanks to the rapid advances of molecular techniques. The great availability of specimens together with molecular methods make the archival tissues the ideal material for clinical and molecular retrospective analysis, which can accelerate the process of translation of the basic molecular cancer biology knowledge into clinical practice (241).

Among functional genomics approaches, proteomics plays a pivotal role in clinical practice due to its potential in translating detailed molecular phenotypes of disease into diagnostic pathology protocols (39, 60-63). Quantitative protein analyses in AT is necessary in the clinical practice since a precise measurement of the expression level of a specific protein could help clinicians/pharmacologists to better define a more appropriate molecular-targeted therapy.

During my doctorate, I have focused my attention on the application of conventional and innovative proteomic approaches on clinical specimens, in particular on AT.

The first part of my research was devoted to the development of suitable protein extraction protocols from tissues with different pre-analytical conditions. Then, I faced up two aspects of clinical proteomics by using two different clinical case studies: (a) in cervix uteri lesions I evaluated, the suitability of new antibodies against the oncoviral protein E7 in the identification of HPV infection for the first time ever; (b) in brain, colon, lung, and thyroid cancer tissues, I investigated the function of the RECQ1 helicase exploiting it as a possible therapy target.

My research achieved the following goals:

1. The standardization of a suitable protein extraction protocol from FFPE tissues. In fact, our study demonstrates that it is possible to harmonize protein analysis in AT in different European laboratories, by using the same protocols for tissue processing and protein extraction.
2. The optimization of an appropriate protocol for molecular analyses of tissues fixed with FineFix. The possibility to use proteomics approaches also on tissues fixed with formalin alternative reagents has great potential for the future pathology, when the use of formalin will be ban due to its carcinogenetic effects. Moreover, FineFix proves a suitable formalin substitute in clinical practice, because preserves both specimens’ morphology and immunoreactivity.

3. The validation of a new antibody against the E7 oncoviral protein, for the detection of HPV in cervical cancer. This lead us to conclude that E7 might be a suitable specific marker for the diagnosis of cervical cancer (by using both IHC and cytological tests), and could be used also for others HPV linked diseases. The analyses of a battery of E7 MoAbs has allowed to select them also for their helpfulness in differential diagnosis.

4. The proposal of two models for the assessment of the progression risk in patients with LSIL, by using the combination of E7 and L1 immunostaining. This evidence, coming from the evaluation of morphological features together with statistical correlation analysis, lead us to stress the importance to use a panel of biomarkers to better classify cervical lesions.

5. An insightful knowledge of the function of RECQ1 in glioblastoma. In fact, for the first time RECQ1 expression has been studied in glioblastoma cell lines, and demonstrated an important role in its growth and proliferation, maintaining genome stability.

6. The proposal of RECQ1 helicase as therapy target in glioblastoma, because the enzyme expression in this tumour is higher than that in perilesional tissues or in cancers of different origin. These findings lead us to consider RECQ1 a reliable target for the development of new anti-cancer therapies aimed at the elimination of proliferating tumour cells.
Altogether, my results demonstrated that AT could represent a valuable source of material for proteomics research.
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LIST OF PUBLICATIONS

Publications


- Ramiro Mendoza-Maldonado, Valentina Faoro, Sailesh Bajpai, Matteo Berti, Federico Odreman, Marco Vindigni, Tamara Ius, Abdollah Ghasemian, Serena Bonin, Miran Skrap, Giorgio Stanta, Alessandro Vindigni. The human RECQ1 helicase is highly expressed in glioblastoma and plays an important role in tumor cell proliferation. Molecular cancer. 2010; Status: Submitted.


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• M. Donada ScD¹; S. Bonin PhD¹ ²; R. Barbazza MD; **V. Faoro** ScD¹ ²; and G. Stanta, MD¹

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• Ramiro Mendoza Maldonado, Sailesh Bajpai, **Valentina Faoro** and Alessandro Vindigni
  *Defining the role of RECQ1 in human cells*. Poster session at the Workshop "Proteomic Characterization of Macromolecular Complexes Involved in DNA Metabolism", Trieste May 2009

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