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MARCATORI IMMUNOISTOCHIMICI E MOLECOLARI AD IMPATTO PROGNOSTICO E PREDITTIVO DI RISPOSTA ALLA TERAPIA IN AMBITO NEOPLASTICO

IMMUNOHISTOCHEMICAL AND MOLECULAR PROGNOSTIC/PREDICTIVE MARKERS IN NEOPLASTIC DISEASES

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INTRODUCTION

Biological markers or biomarkers are gaining increasing importance in clinical practice. They can be used in many different conditions and for many different purposes. As defined by the Biomarkers Definition Working Group in Bethesda, a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"(1). Biomarkers can be analyzed in the serum, in the body fluids, and in tissue specimens, the last being of particular importance in pathology since all this material is stored in the pathological Archives.

In pathology departments and laboratories, many traditional morphological examinations are carried out, especially in neoplastic diseases. Nevertheless, many of the classical morphological analyses, including staging, grading, vascular invasion and assessment of the surgical margins, are not enough anymore to correctly report a tumor. Several subsequent clinical decisions are made upon biomarkers that the pathological report should evaluate. A paradigm example is represented by the mammary neoplasia, in which a complete evaluation of estrogen and progesterone receptors is of mandatory importance in correctly addressing patients to tamoxifen therapy (2). Many biomarkers are currently under study, and their discovery and subsequent validation may help in better define their patients. Unfortunately, although many markers are discovered, little of them are currently validated for clinical practice. One of the reasons is that many of them showed tremendous variations from the different studies and cannot be consistently applied. An international Committee from the Statistics Subcommitee of the National Cancer Institute-European Organization for Research and Treatment of Cancer has developed guidelines, referred to as REMARK, for the reporting of tumor marker studies (3). In these guidelines, it is clearly expressed the importance of good study design and data quality. Poor study reporting has many negative consequences over the research community as a whole. Inappropriately analyzed studies may deserve disproportionate clinical attention, just because the results are apparently dramatic; on the contrary, carefully designed studies may not attract so much consideration, even if they were appropriately conducted, but did not so produce so impressive results (3). Also technical

problems may obstacle the results in a validation process of a biomarker, and in particular immunohistochemical markers may be affected by preanalytical and analytical problems (4). Preanalytical problems include the time to collection, details of fixation, dehydration steps and conditions for paraffin-embedding. Analytical issues are represented, most of all, by: antigen retrieval techniques, type of detection system, choice of the antibody and material to be used (5).

Biomarkers may be subdivided on the basis of their application:

- As a diagnostic tool: they help in identifying patients with a disease (*diagnostic markers*)
- As a tool for staging a disease or classification of the extent of disease (prostate specific antigen, PSA in the blood)
- As an indicator of disease prognosis (*prognostic markers*)
- As an indicator of response to a specific target therapy (*predictive markers*)(1).

From a technical point of view, in pathology, biomarkers may be classified in:

- Immunohistochemical markers
- Molecular markers

Diagnostic markers

Biomarkers with diagnostic purposes are extremely important in correctly identifying patients carrying the specific disease. Morphological examination may not always reach consistent results, especially in small biopsies, with artifactual changes or in difficult cases. By immunohistochemistry, alpha-methylacyl-CoA racemase (AMACR) expression is currently used as a reliable immunohistochemical diagnostic marker for invasive prostatic carcinoma (6). In the next sections, we will also explain our study upon the evaluation of a new diagnostic immunohistochemical marker for prostatic carcinoma, which was compared to AMACR. Laminin-5 γ2 chain is another important immunohistochemical marker, which may

help in identifying invasiveness in colorectal carcinomas (7), squamous cervical carcinomas (8) and glandular cervical adenocarcinomas (9), especially when there is only focal invasion, with small aggregates of detached neoplastic cells (budding).

At the molecular level, FISH (Fluorescence Is Situ Hybridization) may play an important role in establishing monosomy in different chromosomes, by using multiple centromeric probes. For example, the differential diagnosis between renal oncocytoma and chromophobe renal cell carcinoma may be extremely difficult with the morphology, even in association with immunohistochemistry (cytokeratin 7, S100A1) and histochemistry (Hale's colloidal iron stain). However, chromphobe renal cell carcinoma frequently exhibits multiple losses among whole chromosomes 1, 2, 6, 10, and 17 by FISH (10) and by interphase FISH (11). The more complex karyoptypic abnormalities may then address the diagnosis toward a more malignant neoplasm, i.e. the chromopohe renal cell carcinoma.

Prognostic markers

Ki67/MIB1 is a typical prognostic marker and it helps in better identifying grade and biological behavior in many malignancies, especially for those located in the central nervous system (12).

By gene expression profiling, diffuse large B cell lymphomas (DLBCL) have been extensively studied by Alizadeh et al, who identified two major groups: one group expressed genes of germinal centre B cells ('germinal centre B-like DLBCL'), while the other group expressed genes normally induced during in vitro activation of peripheral blood B cells ('activated B-like DLBCL'); patients with germinal centre B-like DLBCL had a significantly better overall survival than those with activated B-like DLBCL (13). Similar subgrouping has been proposed on the basis of the immunohistochemical results, with comparable findings.

Predictive markers

Paradigm examples are provided by mammary neoplasia, where high expression of estrogen and progesterone receptors correlate with tumor responsiveness to the anti-estrogen tamoxifen (2). Estrogen and progesterone receptors are also prognostic markers, since they are more expressed in many well differentiated neoplasms, with a favorable prognosis. In mammary neoplasias, also the amplification of c-erb-B2 is strictly associated with responsiveness to trastuzumab. Its amplification status is currently determined firstly with an immunohistochemical test, followed by a molecular test for suspicious cases. Molecular analysis for amplification of c-cerb-B2 is usually performed with FISH, even if a new dual ISH base in chromogenic assay has been recently approved by the Food and Drug Administration (FDA).

Mutated EGFR pulmonary adenocarcinomas may benefit form tyrosine –kinase inhibitor (TKI) therapy, such as gefitinib (14) and erlotinib (15).

Molecular pathogenesis: the basis to identify new markers (lesson form the colorectal carcinoma)

The basis for identifying new biological markers is certainly represented by molecular carcinogenesis models. Carcinogenesis is a multistep process in which many mutations occur: activation of oncogenes, inactivation of oncosuppressor genes and altered expression of DNA repair genes. All these molecular modifications determine the loss of growth control from the neoplastic cells and, consequently, the neoplastic transformation. A classic example is represented by colorectal carcinoma and its multistep carcinogenesis. In colorectal carcinogenesis two main molecular pathways are identified (16).

The *first* is the classical APC/β-catenin pathway which is particularly involved in familial adenomatous polyposis and activated in 80% of sporadic colorectal carcinomas; this pathway is characterized by the activation of the Wnt pathway, determines k-ras mutations with EGFR activation among the first events and causes p53 mutations in advanced stages. The morphological counterpart is exemplified by the classic adenoma-carcinoma sequence. One of

the critical target in this first oncogenetic pathway is the possibility to block the EGFR signaling induced by k-ras mutations by using anti-EGFR monoclonal antibodies (cetuximab) (17); so the k-ras status represents an important predictive marker for responsiveness to this drug. Other important factors extensively studied in this pathway are: p53 and VEGF. P53 has been shown to be an important prognostic factor in many studies, summarized by meta-analysis reviews (18). VEGF has been described both as prognostic factor (19;20) and as predictive factor to preoperative radiochemotherapy in rectal carcinomas (21).

The *second* pathway is the so-called microsatellite instability (MSI) pathway, in which the DNA mismatch repair genes are damaged. Deficit in these DNA repair genes causes expansion of microsatellite regions. The familial corresponding disease is the Lynch syndrome. In sporadic forms, this pathway affects approximately 20% of the colorectal carcinomas, mainly mucinous adenocarcinomas. The precursor lesion is frequently the sessile serrated adenoma. These lesions are predominately located in the right colon. Molecularly, this oncogenetic pathway is characterized by activation of TGFβ and BAX, BRAF mutations, MLH1 methylation. In translating these basic concepts into clinical practice, several studies have shown that microsatellite instability is both a prognostic and a predictive marker. A meta-analysis upon 7642 cases has shown that MSI tumors are associated to a better prognosis (22). Microsatellite status is also gaining more and more popularity because of its role as predictive factor to response to adjuvant 5-fluorouracil treatment: a study with 570 cases have attributed to the MSI tumors a less responsiveness to 5-FU treatment (23). We have previously reported the role of MUC2 as a predictive marker of responsiveness to radiochemotherapy in rectal adenocarcinoma (24); MUC 2 is associated with mucinous differentiation and, ultimately, to the MSI.

Colorectal carcinogenesis has been herein described as an example of a carcinogenetic process. The same process may be applied in many other human malignancies, as in oral squamous cell carcinoma (OSCC), the central and most important section in this thesis.

Statistical consideration

Statistical validation of a biological marker is one of the most crucial points in determining the real reproducibility of the analyses and its effective application. In many cases, the statistical analyses are not conducted properly and this may alter the impact of the results. Altman et al. reviewed all papers which included analyses of survival data and were published in *British Journal of Cancer*, *European Journal of Cancer*, *Journal of Clinical Oncology* and *American Journal of Clinical Oncology* in a 3-month period; they found many problems in representing statistical data, in reporting all the variables and not only the p-values, in describing all the parameters in the survival curves and in establishing the cut-off points from quantitative to qualitative variable transformation (25).

Survival curves are certainly the gold standard method in order to evaluate a prognostic marker (26). They can be used to study survival, disease-free-survival or any time-dependent event. With this method the main event on study is time and not the event *per se*. Graphically, survival curves can also give an idea to what is the prognostic impact of any disease. One of the most common applications is the comparison between two (or more) survival curves in two groups of patients: the "log-rank test". It is a simple and direct test which may be very useful in comparing the survival curves between two groups. However, the best analysis would be taken if the two groups would be *randomized* (27). Randomization means that the two groups are almost equal, expect for the only one difference that we want to analyze. One of the most common mistakes in the medical history is the evaluation of Salk vaccine against polio in 1954 (28). The study was designed as follows (28).

> *The plan of procedure announced by the National Foundation for Infantile Paralysis and its Advisory Committee was to administer vaccine to children in the second grade of school; the corresponding first and third graders would not be inoculated but would be kept under observation for the occurrence of poliomyelitis in comparison with the inoculated second graders. This has been designated the "Observed Control" study.*
>
> *In observed areas where only those second grade children whose parents requested participation were vaccinated, the problem of establishing the control population was more complex.*

In this study, even if this was one of the biggest trials worldwide at that time, the concept of randomization was totally lacking. Only the second year school children whose parents gave consent were vaccinated and the first and third year of all children represented the control group. The main problems were two. Firstly, the groups were composed of children of different ages; secondly, children from a poor social background were more exposed to polio antigen and their parents more favorable to assign them to the vaccine. So children who were vaccinated were at higher risk to develop the disease. The results showed paradoxically that Salk vaccine enhanced the risk of developing polio disease! This episode underlines the importance of a correct randomization in comparing two groups. Ideally, the two groups should be equal and differ *only* for the characteristic we would like to study. Unfortunately, this situation is very difficult to realize in the clinical practice, but enormous problems may be present when there are many differences between the two groups.

When comparing more variables at the same time, a common procedure is to evaluate survival function with multivariate analysis; the most common method is the use of Cox regression (26). The different variables used in the multivariate analysis should be independent one form the other. Alternatively, many problems and confounding factors may develop.

AIM S OF THE PROJECT

The aim of the project was to identify immunohistochemical and molecular markers which may be useful in correctly identify neoplastic diseases.

We subdivide the work in three main sections:

1) IGFBP2 as a diagnostic marker in prostatic carcinoma.
2) Heparan-sulfate proteoglycans as prognostic markers and their predictive role to responsiveness to adjuvant radiotherapy in oral squamous cell carcinomas.
3) EGFR as predictive markers for responsiveness to tyrosine-kinas inhibitors in pulmonary adenocarcinomas.

IGFBP2 AS A DIAGNOSTIC MARKER IN PROSTATIC ADENOCARCINOMAS

Insulin-like growth factors (IGFs) and insulin-like growth factor binding proteins (IGFBPs) play a central role in cellular growth, and in normal and neoplastic development.

IGFBP2 has been shown to be hyper-expressed in many human malignancies, including ovarian carcinoma (29), colorectal carcinoma (30), hepatocellular carcinoma (31) and neuroblastoma (32). In addition, IGFBP2 has been shown to be highly expressed in glioblastoma, both genetically and immunohistochemically (33). IGFBP2 has even been considered to be a therapeutic target in neoplastic cell lines derived from breast carcinoma, both directly (34) and by modulation of the immune system (35). Furthermore, serum levels of IGFBP2 are reduced in mammary carcinoma (36) and increased in ovarian carcinoma (37), suggesting its possible role as a serological marker for early diagnosis.

In prostatic tissues, genetic profiling studies documented that IGFBP2 was among the genes overexpressed in malignant lesions in comparison to normal cases (38-40). Immunohistochemically, IGFBP2 has been found to be highly reactive in prostatic adenocarcinoma (PAc) (41-43). IGFBP2 has been proposed as a serum prognostic marker for patients affected by PAc (44). A significant association between elevated serum levels of IGFBP2 and the presence of PAc, especially when it is in advanced stages (45-47), has been documented. Yu et al. have shown that serum levels of IGFBP2 were higher in patients with remission than in patients with relapse (48). However, Roddam and colleagues (49) and a meta-analysis conducted by Rowlands et al. (50) did not find a strong association between IGFBP2 serum levels and prostate cancer risk.

Nevertheless, presently IGFBP-2 is rarely applied in routine diagnoses. Alpha-methylacyl-CoA racemase (AMACR) is currently used as an immunohistochemical marker for PAc, especially in biopsies with small acinar lesions suspicious for malignancy (51). However, AMACR is frequently expressed in high-grade prostatic intraepithelial neoplasia (HG-PIN) and, to some extent, also in some benign lesions (52), thus sometimes making the

interpretation of the immunohistochemical results difficult. Therefore, it would be important to find other possible diagnostic markers which could help in the diagnosis of malignancy.

The aim of the study was to evaluate the diagnostic value of IGFBP2 expression in normal epithelium, HG-PIN and PAc, both in patients hormonally untreated and in patients having undergone complete androgen ablation. Results were compared with Alpha-methylacyl-CoA racemase (AMACR).

Materials and methods

Sixty prostatectomy specimens were utilized in this study. The specimens represented the following three groups:

- Group 1: 10 consecutive simple prostatectomy specimens from patients with bladder outlet obstruction due to benign prostatic hyperplasia;
- Group 2: 40 consecutive radical prostatectomy specimens with prostatic carcinoma from patients hormonally untreated before surgery. Preoperative biopsies were available for all the cases;
- Group 3: 10 consecutive radical prostatectomies with prostatic carcinoma from patients who underwent complete androgen ablation three months before surgery. Preoperative biopsies were available in all cases.

The cases of Groups 1 and 2 were retrieved from the files of the Section of Anatomic Pathology "Marcello Malpighi" of the University of Bologna, whereas those of Group 3 were from the United Hospitals-Polytechnic University of the Marche Region, Ancona. All cases had been fixed in 10% buffered formalin and paraffin embedded. Five mm thick sections were stained with hematoxylin and eosin (H&E). For the purpose of this study, the slides of all cases of the three groups were re-examined by two of the authors (AAS and MPF). The cases of Group 1 did show neither PAc nor HG-PIN. For the cases of Groups 2 and 3, the samples with PAc were from the peripheral zone. The pathological stage was based on the 7th 2009 revision of the TNM (53). The Gleason score of the cancers of Group 2 was based on the ISUP 2005 modification (54). Due to the neoadjuvant therapy, the Gleason grading

system was not applied to the cases of Group 3. The presence of HG-PIN was recorded for the cases of Groups 2 and 3.

Immunohistochemistry

Immunohistochemical analysis for IGFBP2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution 1:100) and for AMACR (Cell Marque, Rocklin, CA, U.S.A., dilution 1:100) was performed. Antigen retrieval was obtained by pre-treatment in W-CAP citrate buffer pH 6.0 for IGFBP2 and in W-CAP TEC buffer pH 8.0 for AMACR (Bio-Optica Milano SpA, Milan, Italy) at 98°C for 25 minutes. Inhibition of endogenous peroxidases was performed in 3% H_2O_2 solution. After rinsing the slides in phosphate-buffered saline (PBS) 1x solution with 0.1% solution of detergent Tween 20 (phosphate-buffered saline–Tween 20; Bio-Optica), the sections were incubated in a humid chamber at room temperature for 5 minutes with Ultra V Block solution (Ultravision LP, LabVision Corporation, Thermo Fisher Scientific Inc. Fremont, CA, U.S.A.). They were subsequently incubated in the humid chamber at 4°C for 6 hours with primary antibody for IGFBP2 and at room temperature for 1 hour with primary antibody for AMACR. Sections were then washed in buffered solution and incubated in the humid chamber at room temperature for 20 minutes with primary antibody enhancer solution (Ultravision LP, LabVision Corporation). After several washes in buffered solution, the sections were incubated for 30 minutes in horseradish peroxide (HRP) (Ultravision LP, LabVision Corporation) polymer solution. Reaction was revealed with diaminobenzidin (DAB) solution for 3 minutes and counterstained with hematoxylin.

Evaluation of immunohistochemistry

For each immunohistochemical marker, the percentage of positive cells was calculated in prostate cancer, HG-PIN and normal looking epithelium in Groups 2 and 3 as well as in normal tissue in Group 3. In order to consider not only the percentage of positive neoplastic cells but also their staining intensity, we also estimated the immunohistochemical score, according to McCarthy's scoring system, originally performed on breast neoplasia (55) and

subsequently also applied to prostate carcinoma (56). The immunohistochemical score was calculated as follows: percentage of positive neoplastic cells multiplied by the staining intensity (0: none; 1: weak; 2: moderate; 3: strong). It ranges from 0 to 300. The mean and standard deviation (SD) were determined for the percentages of positive cells and their immunohistochemical scores for both IGFBP2 and AMACR in the three groups.

Statistics

Statistical analysis was carried out with the statistical package SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, U.S.A.). It included ROC (receiver-operator characteristic) curves, the Wilcoxon signed rank test and the Spearman rank test. The differences between the groups were considered statistically significant at a value of $p<0.05$.

Results

1) Group 1 (patients with bladder outlet obstruction)

The ducts and acini of all cases of Group 1 with the exception of one were negative for IGFBP2. A weak positivity for IGFBP2 was seen in urothelial metaplasia and periurethral glands. Stromal and endothelial cells were negative. An identical staining pattern was seen in the normal looking ducts and acini of Groups 2 and 3. Among normal ducts and acini, scattered cells were intensely stained (data not shown). In deeper sections, the cells in the same location were Chromogranin A positive and thus interpreted as neuroendocrine (NE) cells.

As far as AMACR was concerned, normal tissue in the three groups was negative, both in biopsies and in surgical specimens.

2) Group 2: Patients hormonally untreated before surgery

Prostate cancer

IGFBP2 was positive in the cytoplasm of all 40 cases of PAc, both in the preoperative biopsies and in the surgical specimens. The percentage of neoplastic cells immunoreactive for IGFBP2 ranged from 10 to 90% in the biopsies (mean 66%, SD 22%) and from 10 to 80% in the surgical specimens (mean 59%, SD 21%). The immunohistochemical score for IGFBP2 ranged from 10 to 270 in the biopsies (mean 152, SD 82) and from 20 to 240 in the surgical specimens (mean 142, SD 68).

AMACR was positive in all cases (40/40) of PAc. The percentage of neoplastic cells positive for AMACR ranged from 20 to 90% in the biopsies (mean 80%, SD 14%) and from 50 to 90% in the surgical specimens (mean 79%, SD 12%), while the immunohistochemical score ranged from 20 to 270 in the biopsies (mean 210, SD 63) and from 50 to 270 in the surgical specimens (mean 205, SD 56).

No significant correlation was found between IGFBP2/AMACR immunohistochemical scores and the Gleason score, neither in the biopsies (ρ=-0.52 for the Gleason score and p=0.750 for the IGFBP2 immunohistochemical score; ρ=-0.64 for the Gleason score and p=0.693 for the AMACR immunohistochemical score) nor in the surgical specimens (ρ=-0.89 for the Gleason score and p=0.585 for the IGFBP2 immunohistochemical score; ρ=0.001 for the Gleason score and p=0.994 for the AMACR immunohistochemical score). In the surgical specimens, no correlation was found between IGFBP2/AMACR immunohistochemical scores and the corresponding pathological stage (ρ=-0.245 for the stage and p=0.312 for the IGFBP2 immunohistochemical score; ρ= -0.156 for the stage and p=0.523 for the AMACR immunohistochemical score).

HG-PIN

In HG-PIN, a subtle positivity for IGFBP2 was detected in all but 1 biopsy and in all but 4 surgical specimens. The percentage of positive HG-PIN cells for IGFBP2 ranged from 10 to

70% (mean: 25%; SD: 25%) in the biopsies and from 5 to 80% (mean: 18%; SD: 20%) in the surgical specimens. The immunohistochemical score ranged from 5 to 100 (mean: 47; SD: 52) in the biopsies and from 5 to 160 (mean: 39; SD: 47) in the surgical specimens.

The positivity for AMACR in HGPIN was observed in all but 1 biopsy and in all but 1 surgical specimen. The percentage of positive HG-PIN cells for AMACR ranged from 10 to 90% (mean: 55%; SD: 29%) in the biopsies and from 10 to 80% (mean: 34%; SD: 26%) in the surgical specimens, while the immunohistochemical score for AMACR ranged from 10 to 270 (mean: 114; SD: 77) in the biopsies and from 10 to 240 (mean: 76; SD: 71) in the surgical specimens.

Statistical analysis

According to the ROC curve analysis, by examining the percentages of positive neoplastic cells, the overall accuracy (as expressed by the area under each curve) in detecting invasive PAc vs. HG-PIN was higher for IGFBP2 than for AMACR. The area under the ROC curve was higher for IGFBP2 than for AMACR, both in the biopsies (0.914 for IGFBP2 and 0.787 for AMACR) and in the surgical specimens (0.906 for IGFBP2 and 0.887 for AMACR).

Cut-off values for IGFBP2 positivity in discriminating PAc vs. HG-PIN were determined if greater than or equal to 25% of the lesional cells (sensitivity: 0.950, 1-specificity: 0.421 in the biopsies; sensitivity: 0.875, 1-specificity: 0.211 in the surgical specimens). A lesion can be then considered highly suspicious for HG-PIN when less than 25% of the lesional cells are positive for IGFBP2. Cut-off values for AMACR were not identified since they were more difficult to determine and generally higher, the overall performance of the test also being lower than for IGFBP2.

According to the Wilcoxon signed rank test, by examining both the percentages of positive neoplastic cells and their corresponding immunohistochemical scores, IGFBP2 in comparison to AMACR revealed fewer neoplastic cells not only in invasive PAc, but also in HG-PIN. In invasive PAc, IGFBP2 detected fewer neoplastic cells than AMACR, both in the biopsies ($Z=-3.213$, $p=0.001$ by percentage of positive neoplastic cells; $Z=-3.006$, $p=0.003$ by their

immunohistochemical scores) and in the surgical specimens (Z=-4.127, p<0.001 by examining the percentage of positive neoplastic cells; Z=-4.015, p<0.001 by immunohistochemical score). A similar reduction was also observed in HG-PIN, both in the biopsies (Z=-3.595, p<0.001 by percentage of positive neoplastic cells; Z=-3.600, p<0.001 by immunohistochemical score) and in the surgical specimens (Z=-3.001, p=0.003 by percentage of positive neoplastic cells; Z=-2.760, p=0.006 by immunohistochemical score).

3) Group 3: Patients who underwent androgen ablation before surgery

Prostate cancer

Immunohistochemical expression of IGFBP2 in PAc was detected in all (10/10) cases in the biopsies and in 9 out of 10 in the surgical specimens (Figure 1). In particular, the percentage of neoplastic cells immunoreactive for IGFBP2 ranged from 10 to 90% (mean 41%, SD 26%) in the biopsies and from 5 to 70% in the surgical specimens (mean 24%, SD 25%). The immunohistochemical score ranged from 10 to 180 in the biopsies (mean 76, SD 69) and from 10 to 210 in the surgical specimens (mean 52, SD 69).

Immunoreactivity for AMACR was observed in PAc in all (10/10) cases, in the biopsies and the surgical specimens. The percentage of neoplastic cells positive for AMACR ranged from 50 to 90% in the biopsies (mean 79%, SD 12%) and from 10 to 90% in the surgical specimens (mean 48%, SD 28%), while the immunohistochemical scores varied from 100 to 270 in the biopsies (mean 184, SD 57) and from 10 to 270 in the surgical specimens (mean 105, SD 75).

HG-PIN

HG-PIN was seen in 2 biopsies and in 5 surgical specimens.

Discussion

The results obtained in the present study showed that IGFBP2 is consistently positive in PAc, while it is negative in benign prostatic tissues.

Three other studies have documented consistent immunoreactivity for IGFBP2 in invasive PAc (57-59). However, differences have been noted in examining IGFBP2 expression in HG-PIN. The current investigation shows that IGFBP-2 is expressed in HG-PIN, but at a lower level than in PAc. Tennant and coworkers reached a similar conclusion by examining 28 prostatectomy specimens with adenocarcinoma; they identified weak immunoreactivity for IGFBP2 in normal epithelium, moderate staining in PIN and strong or very strong expression in adenocarcinoma (60). In another study conducted by the same group on 24 prostatic specimens (with 20 adenocarcinomas), comparable results were obtained since IGFBP-2 immunoreactivity increased from the normal through the premalignant (i.e., HG-PIN) and into the malignant states (i.e., PAc) (61). Richardsen et al. confirmed negativity or very weak expression in normal epithelium and in benign prostatic hyperplasia (62); however, they did not find any differences between HG-PIN and carcinoma, and they described the overexpression of IGFBP2 in both HG-PIN and carcinoma; in different cases, the intensity varied from weak to moderate to strong, and the pattern varied from diffuse granular staining to strong cytoplasmic staining (63).

The present data failed to find any correlation between IGFBP2 expression and Gleason grade or tumor stage. This is comparable to the three above-mentioned studies (64-66). However, considering total IGFBP2 RNA expression in neoplastic tissue, Figueroa et al. detected significantly higher RNA expression of IGFBP-2 in tumors with a high Gleason score in comparison to tumors with a low score and benign tissue (67).

The present study shows that IGFBP-2 is strongly expressed in the NE cells present in benign prostatic glands. Cells in a similar location do not express AMACR. This finding is similar to that seen previously by Richardsen et al (68). Furthermore, Tennant et al. described very strong immunoreactivity in scattered stromal cells (69), which may represent what we interpreted as NE cells. Previous studies suggested a role of IGFBP2 in stimulating proliferation of prostatic cells (70;71). It is not easy to understand the meaning of IGFBP2

expression in NE prostatic cells, but it may represent a further proof of IGFBP2 involvement in growth stimulation.

In cases of Group 3 (i.e. patients following complete androgen ablation), the expression of IGFBP2 was consistently detected in the initial diagnostic biopsies, but markedly reduced in the surgical specimens after hormonal treatment. The expression of AMACR was also reduced, but at a lower level than that of IGFBP2. Interestingly, androgen ablation did not affect IGFBP2 expression in the NE cells, while AMACR remained negative in the NE cells.

This is among the first studies to document the immunoreactivity for IGFBP2 of PAc following complete androgen ablation. Bubendorf et al. demonstrated that IGFBP2 was genetically overexpressed in a hormone refracting cell line of prostate cancer; on a tissue microarray, they also observed consistent immunoreactivity for IGFBP2 in all tumors which had developed a recurrent tumor during androgen deprivation therapy (72). Similar conclusions were noted by Kyiama et al., who found that IGFBP2 mRNA and protein levels increased 2–3-folds after androgen withdrawal in LNCaP (an androgen-sensitive human prostatic carcinoma cell line); they also identified increased IGFBP-2 immunohistochemical levels after castration using a human prostate tissue microarray of untreated and posthormone therapy treated prostatectomy specimens (73). Other experimental studies on cancer cell lines showed that stimulation by IGFBP2 had a potent stimulatory effect on the growth of LAPC-4 (an androgen-dependent cell line) prostate cancer cells, this effect being more pronounced in the absence of androgens (74). Inman et al. investigated the serum level of IGFBP2 following androgen ablation (75) and they found that high serum levels of IGFBP2 were associated with a better prognosis in patients who received hormonal neoadjuvant therapy and with a worse prognosis in patients who did not receive any preoperative treatment (76). All these findings may support a possible interaction between IGFBP2 and the androgen receptor system.

All these results lead to two considerations. One is that IGFBP2 is a good immunohistochemical marker in the identification of PAc. This concept is also sustained by the role played by IGFBP2 as a serum marker (both diagnostic and prognostic) for PAc (77-83). The other is the fact that IGFBP2 could be involved not only in the transition from HG-

PIN to PAc but also, through the action of the NE cells, in the progression of PAc towards an androgen-independent phase, as demonstrated in those cases with NE differentiation (84-86).

In conclusions the principal findings in the current study were:

- IGFBP2 is not expressed in the normal-looking tissue of the transition and peripheral zones;
- IGFBP2 is expressed in the cytoplasm of untreated PAc and, to a lesser extent, in HG-PIN
- IGFBP-2 is also expressed in PAc and HG-PIN following complete androgen ablation, but to a lesser extent than in the untreated neoplasms;
- IGFBP2 expression in the untreated specimens is lower in HG-PIN than in invasive PAc. This finding can be of diagnostic help especially in thin pre-operative needle biopsies, when a small amount of tissue is available for immunohistochemical stainings.
- Neuroendocrine cells present in prostatic glands are immunoreactive for IGFBP-2 (87).

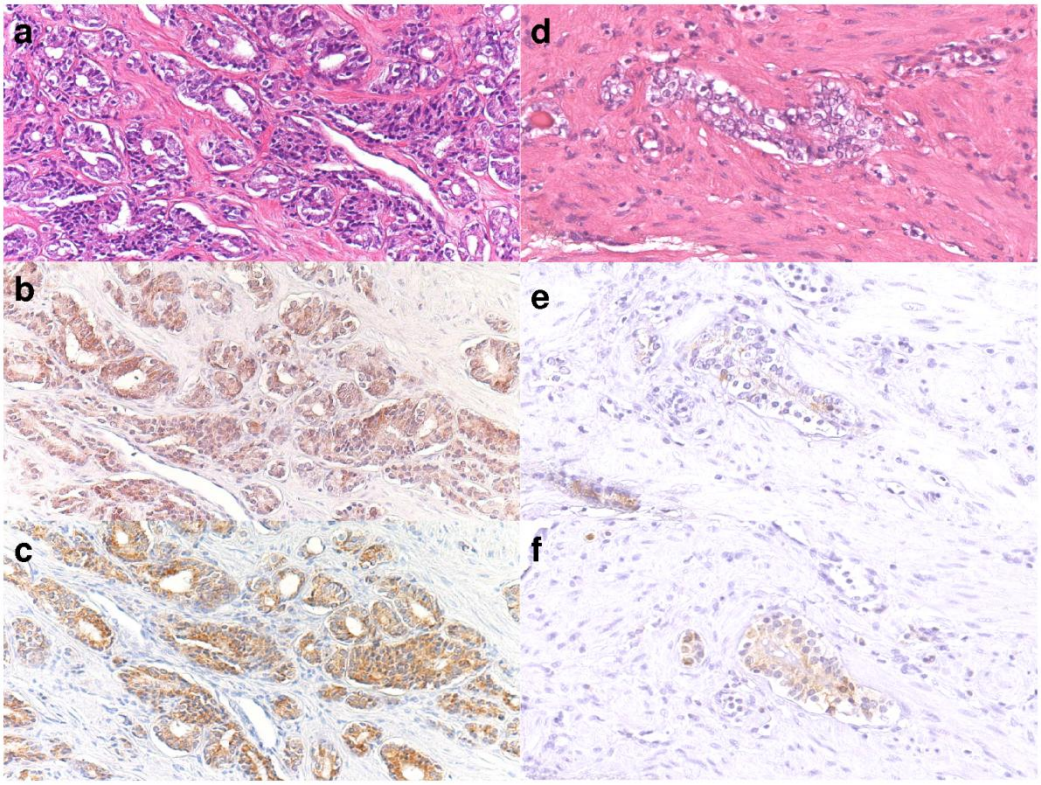


Figure 1. IGFBP2 as a diagnostic marker for prostatic adenocarcinoma (PAc).

Group 3: patients who underwent androgen ablation before surgery.

In biopsies, invasive PAc (a) highly expresses both IGFBP2 (b) and AMACR (c). In surgical specimens, invasive PAc after androgen ablation (d) shows a markedly lowered expression of IGFBP2 (e) while AMACR immunoreactivity is still detectable (f).

HEPARAN SURFACE PROTEOGLYCANS IN ORAL SQUAMOUS CELL CARCINOMAS AS PROGNOSTIC MARKERS AND THEIR PREDICTIVE ROLE TO ADJUVANT RADIOTHERAPY

Oral squamous cell carcinoma (OSCC) is a major disease, with estimated 263,000 new cases worldwide per year (88). In pathological evaluation of OSCC, two of the most critical prognostic points are stage (89;90) and Brodman's grade (91). Other important morphological factors that may play an important role at the morphological evaluation are: tumor thickness and depth of invasion (92;93), desmoplastic reaction of the surrounding stroma (94), tumor associated tissue eosinophilia (95), vascular and/or perineural invasion.

Molecular carcinogenesis in oral squamous cell carcinoma is a step by step process, which involves numerous factors (96;97), similarly to the colorectal carcinogenesis described in the introduction section. From this step-by-step process, many factors have been identified, and some of them deserve special attention because of their prognostic value. Prognostic factors extensively studied are: p16 (98), p53 and p63 (99), cyclin D1 (100). Lymph node metastasis is certainly one of the histopathological parameters that primarily affect prognosis, but many cases and especially small tumors still have undetectable nodal disease (101). Despite the recent diagnostic and therapeutic improvements, factors determining metastatic disease are mostly unknown.

Proteoglycans (PGs) are glycoproteins with one or more covalently attached heparin sulfate chains (102). On the basis of their core protein primary structure, they are classified in cell surface PGs, extracellular PGs, and intracellular PGs (103). Cell surface PGs are either integral membrane PGs or are linked to the membrane via a phosphatidylinositol moiety; they appear to serve as receptors for growth factors and other components of the extracellular matrix, for cell-matrix and cell-cell interactions and as receptors for other cell-cell interaction molecules (104). The major cell surface PGs are syndecans and glypicans (105;106). The syndecan (SYN) family comprises four integral membrane proteins, named SYN-1 to SYN-4 (107). In the glypican (GPC) family there are six family members, known as GPC-1 to GPC-6 (108). Another important cell surface proteoglycan is NG2 (109;110).

Several studies have demonstrated the important role played by cell surface PGs in promoting cell growth and development in human development and in neoplastic events (111;112). In OSCC, immunohistochemical investigations have shown that SYN-1 staining intensity is associated with keratinocyte differentiation and clinical outcome, both in epithelial neoplastic cells (113;114), and in the stroma (115). SYN-2 may function as a cell surface receptor in highly migratory tumor cells (116). Several studies suggested that GPC-3 could act as a tumor suppressor gene (117;118). In culture cell studies, NG2 expression is related to tumor initiations and growth rate, predisposing to poorer prognosis (119).

Aim of the present study is to define the molecular and immunohistochemical expression of cell surface PGs in OSCC, both in epithelial neoplastic cells and in the accompanying stroma, and to establish possible relationship with the clinical outcome.

Materials and methods

Patient selection

A total of 150 cases of OSCC were obtained from the files of the Departments of Surgical Pathology of the Universities of Bologna and Parma (Italy). Patients were surgically treated by three groups of Maxillo-Facial surgeons from Bologna (University of Bologna at Polyclinic S.Orsola-Malpighi and Bellaria Hospital) and Parma (University of Parma), applying the same surgical procedures.

Criteria of selections were the follows: a) all cases were primary OSCC (assessed by pre-operative biopsy), not previously treated by radio or chemotherapy; b) fresh frozen tissue was available for molecular studies.

Follow up information was available in 93 patients for a period ranging from 6 to 34 months (mean: 19, SD: 7).

Adjuvant therapies

In selected patients, especially in those affected by advanced disease, adjuvant postoperative treatments were performed. Chemotherapy consisted of 5-fluorouracil and cisplatinum. Radio therapy was composed of 60 Gy administered in 30-33 sections.

Tissue microarray (TMA) construction

From each case, the block containing representative neoplastic tissue was selected. In order to have uniform immunohistochemical characterization of all cases, tissue micro-arrays (TMAs) were constructed. TMA construction was performed following a previously described procedure (120-122). Briefly, a new cut haematoxylin and eosin-stained section was obtained from each paraffin block and used to define diagnostic areas. Subsequently representative 0.6 mm cores were obtained from each case, and inserted in a grid pattern into a recipient paraffin block using a tissue arrayer. Cases were considered representative when at least 50% of the section was composed of neoplastic tissue. For each case, the core with the highest percentage of tumor cells stained was used for analysis.

Immunohistochemistry

Sections (4 µm) were cut from TMA and stained with the antibodies listed in Table 1. Immunohistochemistry was performed as follows: dewaxing and antigens unmasking occurred simultaneously with the solution W-Cap TEC buffer pH 6 or W-Cap TEC buffer pH 8 (Bio-Optica, Milan, Italy) for 25 minutes at 98° C. The endogenous peroxidases inhibition was performed by 10 minutes incubation with H2O2 (3% in H2O), washing in distilled water and incubating for 5 minutes with Blocking Solution (LabVision, Fremont, CA, USA) to induce the non-specific binding sites saturation; both steps occurred at room temperature. Primary monoclonal antibodies, listed in Table 1, were applied on sections for 60 minutes at room temperature. Following, chromogenic detection was performed using the UltraVision Detection System (LabVision, Fremont, CA, USA), which provided incubation with Antibody Enhancer for 20 minutes followed by HRP-Polymer for 30 minutes. Finally, DAB chromogen

(Dako, Carpenteria, CA, USA) was applied for 3-5 minutes and sections were counterstained with hematoxylin after washing with water.

Antibody	**Clone**	**Manufacturer**	**Dilution**	**Antigen Retrieval**
SYN-1	*MI15*	*Dako*	1:100	W-CAP pH 6
SYN-2	*1F10/B8*	*Santa Cruz Biotechnology*	1:50	W-CAP pH 8
SYN-3		*Sigma Aldrich (Powered by AtlasProtein)*	1:50	W-CAP pH 8
SYN-4	*H-140*	*Santa Cruz Biotechnology*	1:100	W-CAP pH 8
GPC-1	*4D1*	*Millipore*	1:50	W-CAP pH 6
GPC-3	*1G-12*	*Biomosaics*	1:250	W-CAP pH 6
GPC-4 (aa 54-66)		*Immundiagnostik*	1:500	W-CAP pH 8
GPC-6		*Sigma Aldrich (Powered by AtlasProtein)*	1:20	W-CAP pH 8
NG2	*132.38*	*Sigma Aldrich*	1:50	W-CAP pH 6
4D1 surnatant	*Handle Made*		1:5	W-CAP pH 8

Table 1. List of all antidodies used. Legend: SYN syndecan, GPC glypican.

Immunohistochemical evaluation

The evaluation and scoring of the immunohistochemical results were performed with a light microscope (Nikon) at a magnification of 40X. Each antigen expression was semiquantitatively evaluated and scored as follows:

- Score 0 (-) = no positive cells were detected
- Score 1 (+\-) = <10% of cells were positive
- Score 2 (+) = 10-50% of cells were positive
- Score 3 (++) = >50% of cells were positive
- Score 4 (+++) = >90% of cells were positive

Statistical analysis

For each case, mean and standard deviation (SD) of all variables was determined. Spearman's rank order correlation (ρ) was calculated among the different immunohistochemical scores and differentiation grade, in order to detect possible relationships among them.

Overall survival and disease-free specific survival were analyzed in both univariate and multivariate analyses. Single factors were analyzed with univariate analysis and the statistical significance was calculated with log rank test. Group of factors were studied together in multivariate analysis and their statistical significance was calculated with Cox regression analysis. In multivariate analyses, many different models were tested, with different combinations. The main models were: 12 independent variables (SYN-1, SYN-2, SYN-3, SYN-4, GPC-1, GPC-3, GPC-4, GPC-6, 4D1 surnatant in the epithelial neoplastic cells, and SYN-1, SYN-2 , GPC-1 in the stroma), 6 independent syndecan variables (SYN-1, SYN-2, SYN-3, SYN-4, in the epithelial neoplastic cells, and SYN-1, SYN-2 in the stroma), 4 independent syndecan variables (SYN-1, SYN-2, in the epithelial neoplastic cells, and SYN-1, SYN-2 in the stroma) . For the validity of the regression models, GPC-4 expression in the stroma was removed from the variables, because it was correlated with GPC expression in the epithelial cells. NG2 was also not included, because too few measurements were performed.

All data were analyzed with SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical significance was taken at p value (two-tailed) less than 0.05.

Results

Clinical and pathological features

Patients were 62 females and 88 males, with age varying from 27 to 93 (mean 62; SD 14). Smoke and alcohol consumption was known in 86 patients, among which 54 were habitual smokers and 44 referred regular alcohol consumption.

With the 2010 TNM staging system (53), cases were classified as follows: 41 pT1, 54 pT2, 13 pT3, and 42 pT4; 88 pN0, 27 pN1, 31 pN2, 2 pNx; 35 stage I, 28 stage II, 27 stage III, 57 stage IV.

Lymph node metastases were detected in 60 cases at presentation, and in 4 cases in the FU; in 2 cases no lymph node was resected.

Follow up revealed local recurrence in 10 cases, 3 cases with lymph node metastasis, 1 case with local recurrence and lymph node metastasis; among all of them, 11 patients were deceased (1 with esophageal carcinoma). In 71 patients no recurrence and/or metastasis was documented.

In the constructed TMA, 3 cases were composed of carcinoma in situ, among them 1 case was composed only of in situ carcinoma; 1 case did not contained sufficient material. Representative sections for all markers under study were available in 148/148 invasive OSCCs.

Immunohistochemical features in invasive carcinoma (148 cases)

SYN-1 (CD138). The staining was predominantly observed with a cell membrane pattern. The epithelial neoplastic cells were positive for SYN-1 in 133/148 cases (Figure 1a), while

the stromal component was positive in 31/148 cases (Figure 1b). Staining was mainly localized in keratinizing neoplastic cells, located at the center of the neoplastic nests.

SYN-2. The staining was predominantly observed with a cell membrane pattern. SYN-2 was positive in the epithelial neoplastic cells in 21/148 cases (Figure 1c). The stromal cells were positive in 108/148 cases (Figure 1d). Staining increased when desmoplastic stroma appeared. Furthermore, SYN-2 marked the thin stromal vessel walls in 45/148 cases.

SYN-3. The staining was predominantly observed with a diffuse cytoplasmic pattern and focal membrane reinforcement. This marker was positive in the epithelial neoplastic cells in 54/148 cases. No reactivity was detected in the stroma.

SYN-4. The staining was predominantly observed with a diffuse cytoplasmic pattern and focal membrane reinforcement. In the epithelial neoplastic cells, SYN-4 was positive in 31/148 cases. No reactivity was detected in the stroma.

GPC-1. The staining was predominantly observed with a diffuse cytoplasmic pattern and membrane reinforcement. In the epithelial neoplastic cells, GPC-1 was positive in 111/148 cases (Figure 2a). Stromal reactivity was strongly seen only in 8/148 cases.

GPC-3. The staining was predominantly observed with a diffuse cytoplasmic pattern and membrane reinforcement (Figure 2b). In the epithelial neoplastic cells, GPC-3 was positive in 27/148 cases. No reactivity was detected in the stroma.

GPC-4. The staining was predominantly observed with a diffuse cytoplasmic pattern. In the epithelial neoplastic cells, GPC -4 was positive in 55/148 cases (Figure 2c). In the stroma, 27/148 cases exhibited diffuse immunoreactivity.

GPC -6. The staining was predominantly observed with a granular cytoplasmic pattern. In the epithelial neoplastic cells, GPC-6 was positive in 54/148 cases. No reactivity was detected in the stroma.

NG 2. The staining was predominantly observed with a nuclear pattern. In the epithelial neoplastic cells, NG2 was positive in 35/36 cases. No reactivity was detected in the stroma.

4 D1 surnatant. The staining was predominantly observed with a granular cytoplasmic pattern. The epithelial component was negative in all 98/98 cases. In the stromal component, 4 D1 was positive only in stromal cells in 18/148 cases (Figure 2d).

Immunohistochemical features in carcinoma in situ

The 3 cases composed of carcinoma in situ showed moderate/diffuse staining for SYN-1 and GPC-1 in the epithelial neoplastic cell. The other markers were negative, both in the epithelial neoplastic cells and in the stroma.

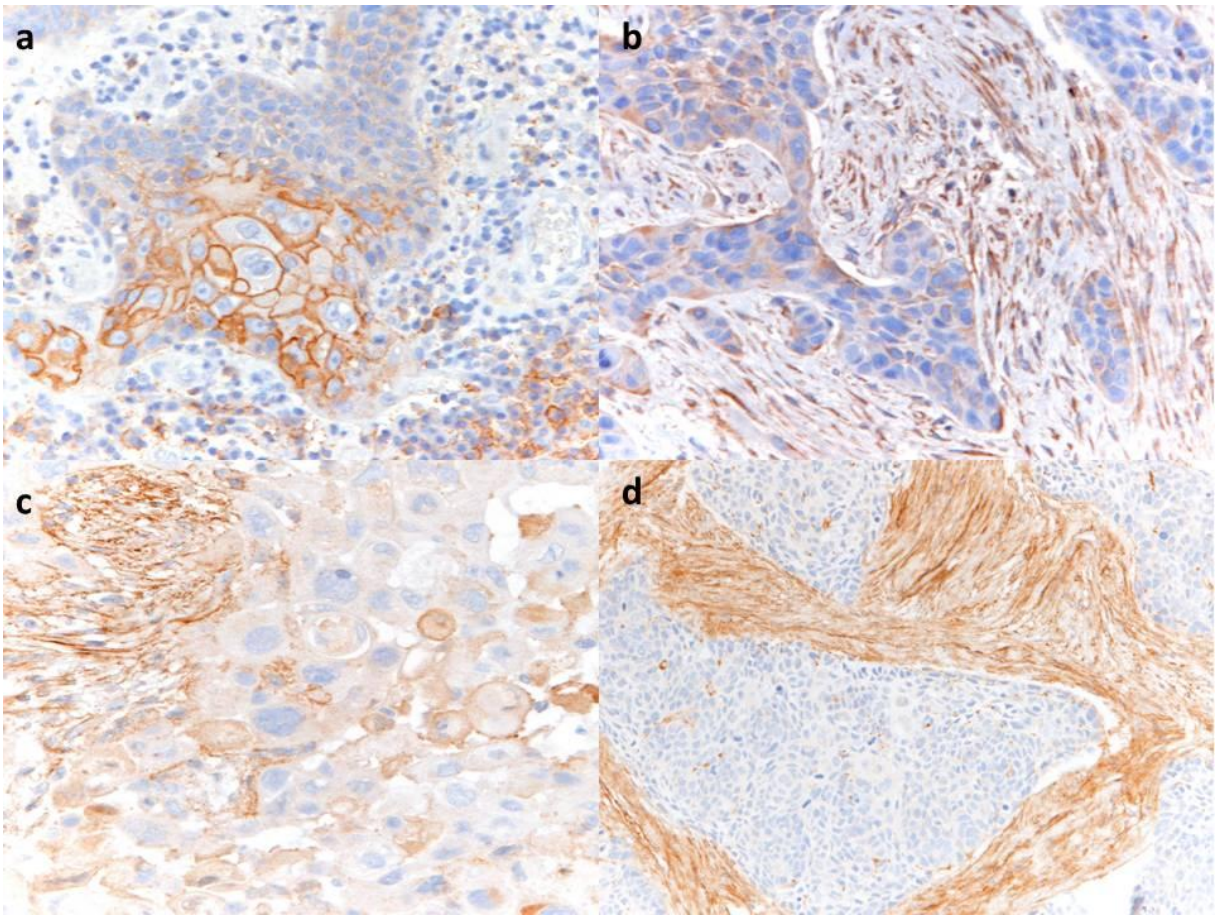


Figure 2. Immunohistochemical expression of syndecans.
Syndecan-1 (SYN-1) immunoreactivity in epithelial neoplastic cells (a) and in stromal cells (b); syndecan-2 (SYN-2) immunoreactivity in epithelial neoplastic cells (c) and in stromal cells (d).

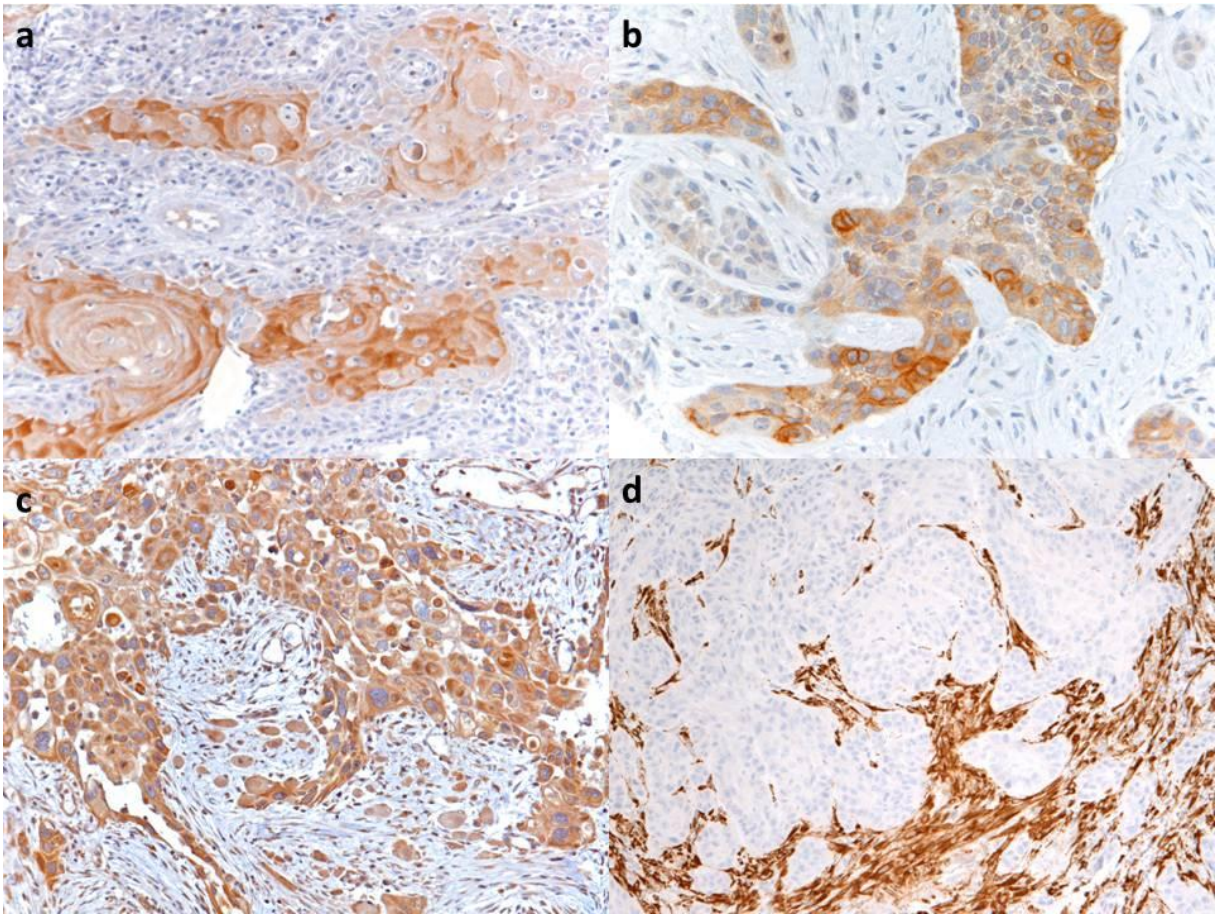


Figure 3. Immunohistochemical expression of glypicans and 4 D1.
Immunohistochemical expression of glypican-1 (GPC-1) (a), glypican-3 (GPC-3) (b) and glypican-4 (GPC-4) (c) in epithelial neoplastic cells; 4D1 surnatant is expressed only in stromal cells (d).

Statistical analysis

Statistical analysis was carried out only in invasive carcinoma (148 cases). The strongest correlation among the immunohistochemical scores was found between GPC-4 expression in the epithelial neoplastic cells and GPC-4 expression in the stroma ($\rho = 0.775$, $p < 0.0001$).

In cases analyzed with all data and available follow-up, survival multivariate analysis with Cox regression model revealed a statistical significance between syndecan 1 expression and reduction of overall survival. In such association we can appreciate 0.046 p-value with 1.967 odds radio. This is the summary of the analysis (Table 2).

	B	**SE**	**Wald**	**df**	**P**	**Exp(B)**
Syndecan 1 (epitelial neoplastic cells)	0.676	0.339	3.973	1	0.046	1.967
Syndecan 1 (stroma)	0.473	0.415	0.011	1	0.917	1.044
Syndecan 2 (epitelial neoplastic cells)	-0.616	0.898	0.471	1	0.493	0.540
Syndecan 2 (stroma)	0.485	0.249	3.787	1	0.052	1.624

Table 2. Summary of the Cox regression model in all patients with OSCC.

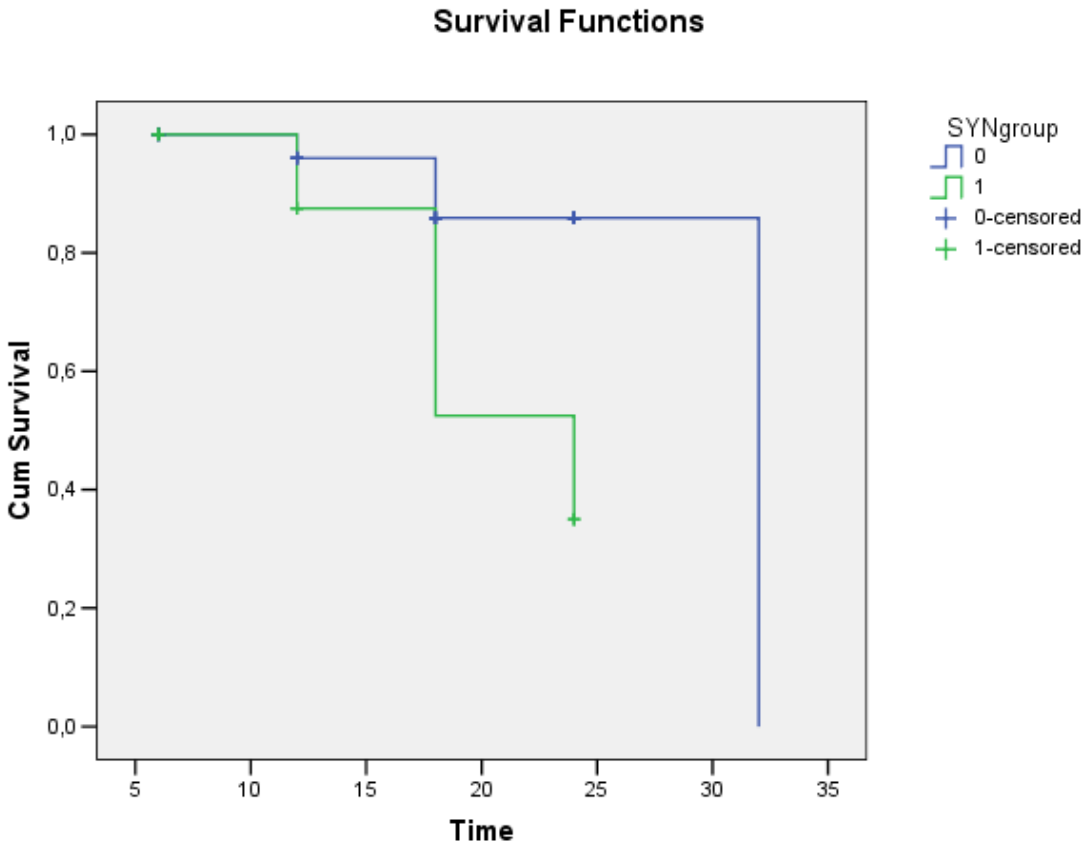
The same analysis, repeated on patients with received subsequent adjuvant radiotherapy (total 44 cases) revealed a stronger association between SYN-1 expression and reduction of overall survival. In such analysis we can appreciate 0.023 p-value with 3.479 odds radio: herein the statistical association is much stronger than the previous one. Then we can reasonably conclude that, in patients submitted to adjuvant radiotherapy, the immunohistochemical expression of SYN-1 in neoplastic cells, significantly decreases the overall survival. This is the summary of the analysis (Table 3).

	B	**SE**	**Wald**	**df**	**P**	**Exp(B)**
Syndecan 1 (epitelial neoplastic cells)	1.247	0.548	5.167	1	0.023	3.479
Syndecan 1 (stroma)	0.319	0.635	0.253	1	0.615	1.376
Syndecan 2 (epitelial neoplastic cells	-0.964	1.049	0.845	1	0.358	0.381
Syndecan 2 (stroma)	0.050	0.325	0.024	1	0.877	1.052

Table 3. Summary of the Cox regression model in patients with OSCC who received radiotherapy.

In order to better clarify the role of SYN-1 alone in patients who received radiotherapy, a univariate analysis of survival was performed. Log-rank test revealed a significant association between SYN-1 score and survival (p=0.001).

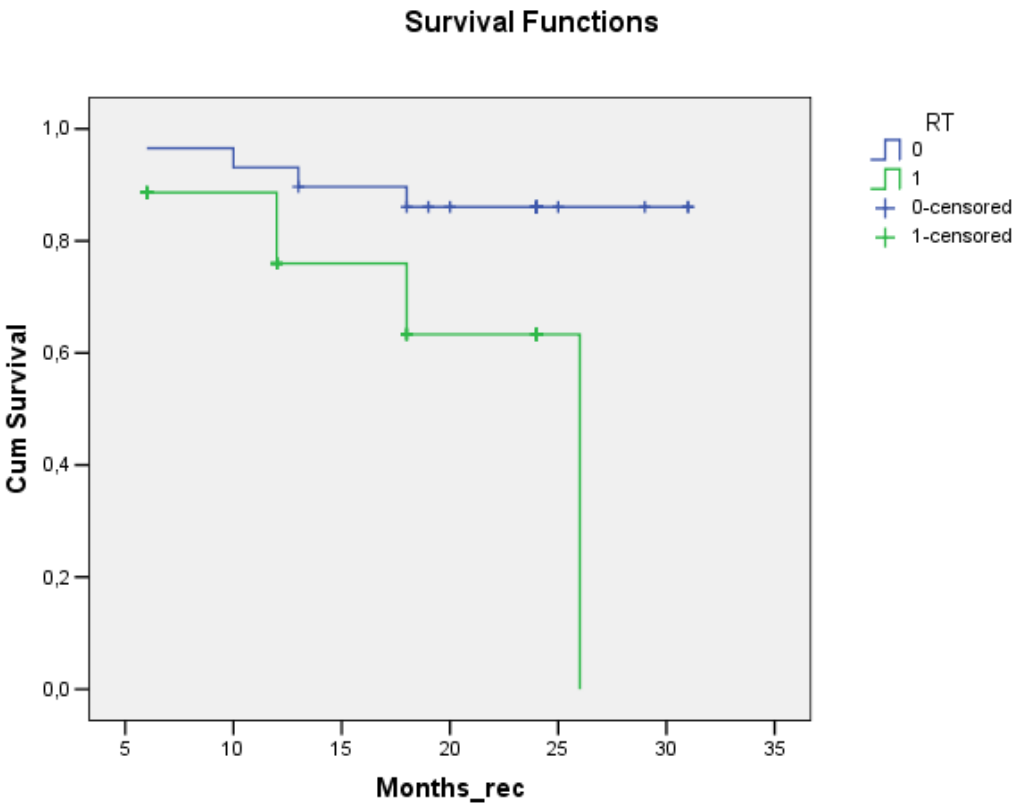
The scoring system in 5 levels, as described before, was important in correctly evaluating the exact percentage of neoplastic cells which were positively stained for SYN-1. However, just for practical purposes, we tried to subdivide the scoring system for SYN-1 immunoreactivity in two major groups: expression more or less than 50% of the neoplastic cell population. With this cut-off, the log-rank test confirmed the statistical significance (p=0.019). Herein we can appreciate this statistical significance with the two overall survival curves.



In this graph we could graphically evaluate that, if SYN-1 expression was more than 50% (*green line*), the survival was significantly reduced. On the contrary, if SYN-1 immunoreactivity was less than 50% (*blue line*), the overall survival was significantly increased.

In summary, such association between SYN-1 and overall survival became stronger after repeating the same analysis only in patients who received radiotherapy. In this view, SYN-1 may be considered not only as a prognostic factor, but also as a predictive factor for responsiveness to adjuvant radiotherapy.

Other interest results were obtained in considering the disease-free survival curves, in examining the role of radiotherapy. We noted a significant reduction in disease-free survival for patients who received adjuvant radiotherapy (p=0.021).



In this graph the green line represented patients who received radiotherapy. This group of patients showed a significant reduction in disease-free survival. This apparently paradoxical

result is explained by the fact that radiotherapy is usually administered in patients affected by OSCC at advanced stage; these patients would certainly display an adverse prognosis in comparison to the other group.

Unfortunately, the role of chemotherapy was not examined, due to the fact that a very small number of patients received this type of treatment. In fact, only 6 patients were treated with this adjuvant chemotherapy; this number was too few for any type of statistical consideration.

Discussion

Cell adhesion molecules, such as integrins, cadherins and cell-surface glycoproteins, are involved in many phases of the cell cycle, in differentiation, migration and in many different stages of neoplastic development (123-126).

The major cell surface PGs are syndecans and glypicans (105;106). The syndecan (SYN) family comprises four integral membrane proteins, named SYN-1, SYN-2, SYN-4 and SYN-4 (107). In the glypican (GPC) family there are six family members, known as GPC-1, GPC-2, GPC-3, GPC4, GPC-5 and GPC-6 (108). Another important cell surface proteoglycan is NG2 (109;110).

Cell surface PGs play an important role, nor only in cellular development, but also in many neoplastic diseases (111;112). In OSCC, some researchers have focused on SYN-1 (also known as CD138) immunoreactivity as a prognostic factor. Inki et al. have studied 29 patients affected by squamous cell carcinoma of the head and neck and they found significant association between SYN-1 immunohistochemical expression and grade of differentiation; overall survival and recurrent-free survival were reduced in patients with low SYN-1 expression (113). Anttonen et al. have analyzed 175 patients affected by squamous cell carcinoma of the head and neck; they have noted important associations between SYN-1 expression and histological grade, stage, tumor size; they also have described a significant reduction of overall survival in patients with reduced (<80%) expression of SYN-1.

Our results are apparently in contrast with these two previous studies, because our data suggested that an increased SYN-1 expression was associated with reduced overall survival. Nevertheless, these two studies considered all squamous cell carcinomas of the head and neck region. In our study, we collected only the tumors of the oral cavity. Furthermore, other studies performed upon tumors in the head and neck area and in other districts showed similar conclusion to ours. For example, Chen et al. revised 157 nasopharingeal carcinomas and found that tumors with SYN-1 expression showed reduction of overall survival in comparison to tumors negative for SYN-1. Barbareschi et al. found an association between high SYN-1 expression and a a more aggressive phenotype in breast neoplasias (127). Other studies found high SYN-1 expression in association with poorer prognosis or aggressive phenotypes, in prostate neoplasias (128), and in thyroid carcinomas (129).

All these previous studies reported conflicting results on the role played by SYN-1 as a prognostic marker. In this view, our data may help in understanding future studies. Furthermore, this association between SYN-1 and prognosis was not only found in all cases with available follow up, but it was much more increased when calculated in patients which underwent radiotherapeutic treatment (p-value 0.023; odds ratio 3.479). This increased association not only confirmed the role of SYN-1 as a prognostic factors, but it introduces also the possibility to consider SYN-1 as a predictive factor for establishing the responsiveness to radiotherapy. To be a confirmed as a predictive role as responsiveness to radiotherapy, a randomized study may be performed.

SYN-1 expression was also studied in the stroma by Mukunyadzi (115), who found an increased expression in invasiveness foci, but without any association with prognosis. We also evaluated the stroma, and found no association with prognosis. Other cell surface PGs have been studied in the literature. SYN-2 has been evaluated in neoplastic cell culture studies and may function as a cell surface receptor in highly migratory tumor cells (116). Some authors have demonstrated that GPC-3 could act as a tumor suppressor gene (117;118) . In culture cell studies, NG2 expression is related to tumor initiations and growth rate, predisposing to poorer prognosis (119). In our study, with all the other markers (except SYN-1) we did not found any statistical association.

The last analysis conducted was the disease-free survival curves upon patients with radiotherapy against patients which received no adjuvant treatment. This analysis was carried out in order to better clarify the two groups of patients; if different results had been discovered, further analyses would have been performed and possible association with PG expression investigated. Nevertheless, even if statistical associations were found, they totally lacked any clinical significance. As explained before, this apparently paradoxical result is explained by the fact that radiotherapy is usually administered in patients affected by OSCCs at advanced stage; these patients would certainly display an adverse prognosis in comparison to the other group. This is an example of a statistically but not clinically significant result.

The last consideration is for the recent perspectives upon the role of HPV infection in the development of OSCC. It is now clear that HPV, and in particular the high risk type 16 (HPV-16), play a central role in the oncogenetic process (130). The OSCCs associated with HPV are predominantly localized in the palatine tonsil and lingual tonsils of the oropharynx and are usually not-keratinizing, mainly of basaloid type. OSCCs associated with HPV affect predominantly young patients, with no other known risk factor for OSCC (alcohol and tobacco) and carry a significant better prognosis. In our data, HPV infection was not analyzed and is currently under investigation in the University of Pavia for another research project. Nevertheless, the cases we selected were mainly localized on the oral cavity and not in the oropharynx. Furthermore, it is becoming more and more difficult to discriminate which is the real role played by HPV in OSCC. HPV is a ubiquitous infection and even its detection may represent only a superimposed infection, especially for the low risk types.

In conclusion our study demonstrated that:

1. SYN-1 is a prognostic factor, since it is significantly increased in OSCCs with poor prognosis, with reduction of overall survival.
2. This association between SYN-1 and overall survival is much stronger in patients with subsequent adjuvant radiotherapy
3. SYN-1 may be a predictive factor of responsiveness to adjuvant radiotherapy.

EGFR MUTATION-SPECIFIC ANTIBODIES IN PULMONARY ADENOCARCINOMA: A COMPARISON WITH DNA DIRECT SEQUENCING

Epidermal Growth Factor Receptor (EGFR) is a trans-membrane receptor with tyrosine kinase activity that plays a central role in regulating cell growth and differentiation, both in normal and neoplastic cells (131). In patients affected by non-small cell lung cell cancer (NSCLC), specific mutations of the EGFR gene correlate with pathological features and responsiveness to tyrosine kinase inhibitors (TKIs) such as gefitinib (14) and erlotinib (15). In EGFR-mutated lung adenocarcinoma, gefitinib is superior to carboplatin-paclitaxel therapy and EGFR mutations strongly predict outcome after therapy (132). Mutational status of the EGFR gene is of thus of primary importance in defining therapeutic decisions (133).

The mutational spectrum of EGFR in lung adenocarcinomas is variable including in-frame deletions, in-frame insertions/duplications and point mutations. The two most common genetic changes are an in-frame deletion in exon 19 at codons 746 to 750 (E746-A750 deletion) and the substitution of leucine 858 by arginine (point mutation L858R) in exon 21 (134). Together, these two mutations account for approximately 90% of the cases and are termed “classical” mutations (135). The gold standard technique for identifying EGFR mutation is direct DNA sequencing of PCR-amplified regions of exons 18, 19, 20, 21 (133) but its clinical application is limited due to problems of tissue conservation and sampling, costs and technical difficulties.

Recently, two novel antibodies that specifically recognize the E746-A750 deletion in exon 19 and the L858R point mutation in exon 21 have been described (136). As immunohistochemistry (IHC) may represent a faster, more economic and more widely applicable alternative to DNA sequencing, the aim of the present study was to compare the two procedures in a series of pulmonary adenocarcinomas.

Materials and Methods

We retrieved from our files all 18 cases of advanced pulmonary adenocarcinomas with EGFR mutations and 15 cases with wild-type EGFR. Specimens were routinely fixed in buffered

formalin and embedded in paraffin. Sections (4 µm) were stained with haematoxylin and eosin for histological diagnosis. All cases were morphologically reviewed and classified according to the most recent IASLC guidelines for lung adenocarcinomas; in each case the prevalent pattern of growth was recorded (137). Immunostaining using a panel of antibodies (138) was performed in selected cases, especially in the metastatic lesions and in the small endoscopic biopsies, to confirm histotype and pulmonary origin of the lesion.

Representative blocks were selected for each case, additional unstained sections were obtained and both molecular and immunohistochemical analyses were performed.

Molecular analysis

Tumor tissue was micro-dissected from formalin-fixed paraffin-embedded (FFPE) sections to obtain samples with at least 50% of neoplastic cells and genomic DNA was extracted. PCR amplification of exons 18, 19, 20, 21 of EGFR was performed using previously described primers (14). Amplicons were sequenced and analyzed on both forward and reverse strands; mutations were verified in two independent experiments.

Immunohistochemical analysis

IHC sections (4 µm) were stained with the following primary antibodies: EGF Receptor, E746-A750del Specific (6B6) at a working dilution of 1/200 and EGF Receptor, L858R Mutant Specific (43B2) at a working dilution of 1/200 (Cell Signaling Technology, Inc, Danvers, MA, USA). Each case was tested with both antibodies. For antigen retrieval, sections were treated with pH 9 Tris-EDTA buffer for 30 minutes in water-bath at 98°C. The slides were developed in diaminobenzidine (DAB) using the HRP Polymer (Ultravision LP Large Volume Detection System; Lab Vision, Fremont, CA, USA) in accordance with the manufacturer's specifications and were counterstained with hematoxylin.

Immunoreactivity was determined with the following scoring system, as previously described (139): 0=no staining or faint staining intensity in < 10% of tumor cells; 1+=faint staining in

>10% of tumor cells; 2+=moderate staining, 3+=strong staining. In differentiating score 1+ from score 2+, we found useful the presence of membrane reinforcement. Cases with faint, diffuse cytoplasmic staining were classified as 1+, while cases with moderate staining and focal membrane reinforcement were classified as 2+. Distinction between 1+ and 2+ is crucial, because the subsequent statistical evaluation considered 1+ as negative and 2+, 3+ as positive for EGFR mutational status. The IHC scoring system is summarized in this Table 4. Cases were further classified on the basis of the pattern of immunoreactivity: patchy or diffuse, comparing different areas of the same slide.

IHC score	**Reactivity**	**Membrane reinforcement**	**Mutational status**
0	no staining or faint staining intensity in < 10% of tumor cells	no	not consistent with mutation
1	faint staining intensity in >10% of tumor cells	no	
2	moderate staining intensity	focal	consistent with mutation
3	strong staining intensity	focal or diffuse	

Table 4. Immunohistochemical scoring system.

Statistical analysis

Sensitivity and specificity of IHC were calculated using the molecular status as reference. The agreement between the two techniques was calculated with Coehn's kappa. In comparing the two techniques, IHC scores 0 and 1+ were considered as negative for mutational status, while IHC scores 2+ and 3+ were interpreted as positive, as already suggested by Kawahara et al. (140). All data were analyzed with SPSS 13.0 for Windows.

Results

We evaluated 33 cases of lung tumours that had been previously analyzed for EGFR mutations. There were 21 females and 12 males, with age ranging from 48 to 78 (mean 62.0 ± 10.6). All cases were lung adenocarcinomas, 23 primaries (5 endobronchial biopsies, 18 pulmonary surgical resections) and 10 metastatic (7 pleural biopsies, 1 hepatic biopsy, 1 axillary lymph node resection, 1 cerebral metastasis resection). The prevalent pattern of growth was acinar in 17 cases (in 3 of them with extensive mucinous features), lepidic in 2, papillary in 3, solid in 11. In 1 case, a small cell neuroendocrine component was associated; another case exhibited focal squamous differentiation.

By conventional DNA sequencing, 12 cases had EGFR mutations in exon 19 and 6 in exon 21. In exon 19, we considered 9 cases with E746-A750 deletion and 3 cases with alternative in-frame deletions: 1 with L747-T751del in homozigotic status, 1 with L747-P753del, 1 with E747-S752del. In exon 21, we examined 5 cases with point mutation L858R, and 1 case with alternative point mutation L861Q+L862L. The tumor with small cell neuroendocrine component exhibited the L858R point mutation in the glandular component. The tumor with squamous cell differentiation was examined in the glandular component and was EGFR wild-type. Furthermore, 15 EGFR wild-type cases were evaluated as negative controls.

Overall, there were 11 cases with strong staining (2+ and 3+ scores), 4 with weak staining (score 1+) and 18 cases that showed no immunoreactivity. Staining was diffuse in 13 cases and patchy in 2 cases. Patchy staining was observed in 1+ cases, whereas in 2+ and 3+ cases the percentage of stained cells was always more than 70%.

The E746-A750del specific antibody detected 6 of 9 cases with E746-A750del mutation (5 with score 3+, 1 with score 2+) whereas it was negative in three cases (2 with score 1+, 1 with score 0) (kappa=0.744, sensitivity: 66.7%, specificity: 100%). The three cases with alternative mutations on exon 19 were negative. Overall, 6 of 12 of cases with exon 19 mutations were identified (kappa=0.560, sensitivity: 50%, specificity: 100%).

The L858R specific antibody correctly classified all five cases with the corresponding gene mutation (4 with score 3+, 1 with score 2+) (kappa=1, sensitivity: 100%, specificity: 100%) (Figure 4). The case with the alternative exon 21 mutation L861Q+L862L was negative. Overall, 5/6 cases with mutations in exon 21 were detected (kappa=0.891, sensitivity: 83.3%, specificity: 100%).

All immunoreactive cases were negative when tested with the second antibody. All 15 EGFR wild-type control cases were negative with both antibodies (100% specificity). Among them, two cases exhibited score 1+ immunoreactivity with the E746-A750del specific antibody, whereas all remaining tumors scored 0.

The overall performance of the two mutation-specific antibodies in the 33 tested cases gave a kappa value of 0.588, with 61.1% sensitivity and 100% specificity (Table 5).

	Sensitivity		Specificity		kappa	
	E746-A750del (6B6)	**L858R (43B2)**	**E746-A750del (6B6)**	**L858R (43B2)**	**E746-A750del (6B6)**	**L858R (43B2)**
Detection of specific mutation	6/9 (66.7%)	5/5 (100%)	100%	100%	0.744	1
Detection of all mutations in the same exon	6/12 (50%)	5/6 (83.3%)	100%	100%	0.560	0.891
Overall	11/18 (61.1%)		100%		0.588	

Table 5.
Summary of sensitivity, specificity and kappa values of EGFR mutation-specific antibodies compared with molecular detection.

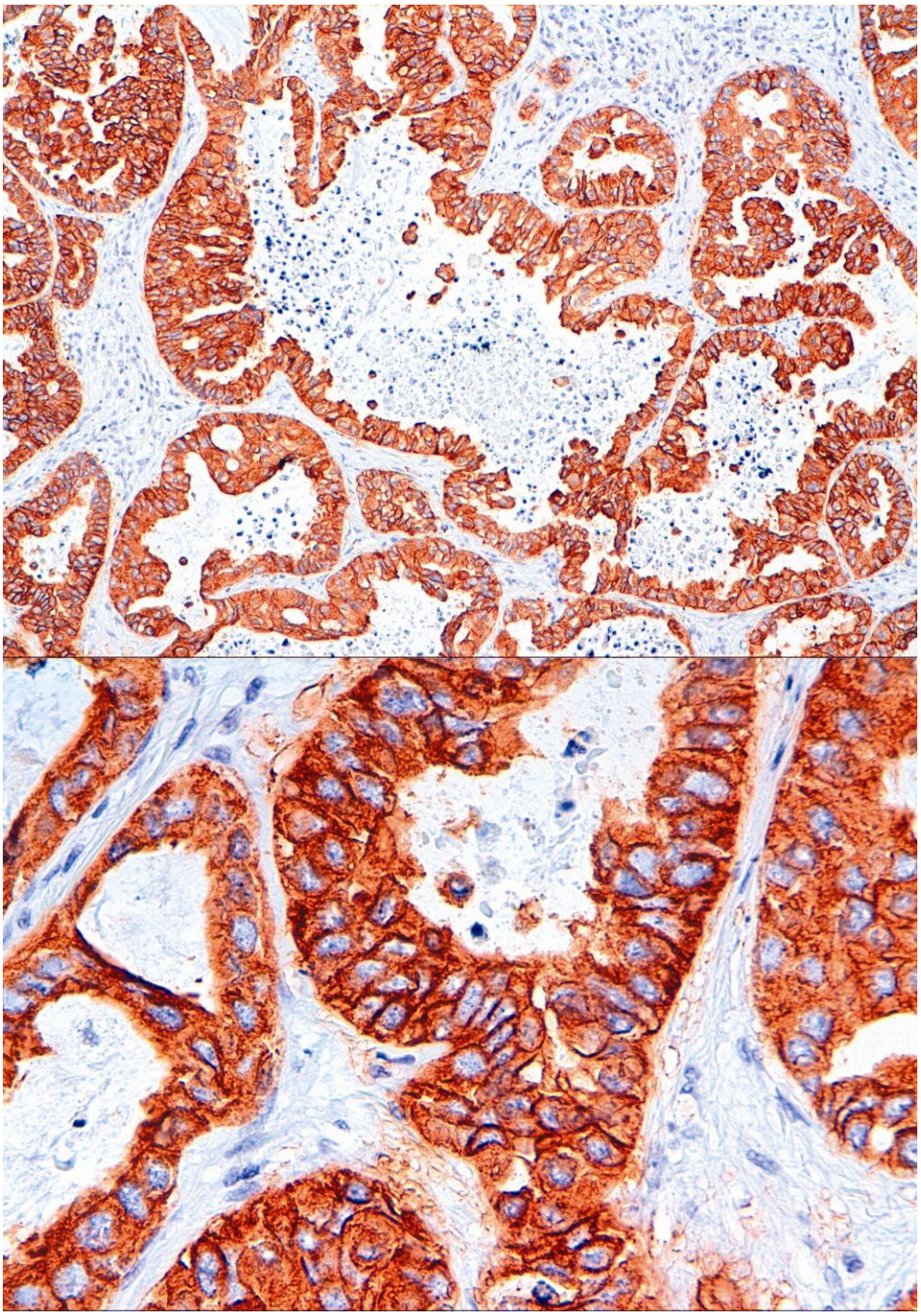


Figure 4. Immunohistochemical expression of EGFR, detecting mutation L858R on exon 21. In case N. 16 the L858R specific antibody strongly and intensely stains the neoplastic glands; membrane reinforcement is detectable (*bottom*) (score 3+, consistent with mutation).

Discussion

The comparison between molecular and immunohistochemical methods of EGFR mutations detection in lung adenocarcinomas demonstrated two main points:

1. the L858R antibody had higher sensitivity than the E746-A750del antibody
2. IHC showed very high specificity (100% in each comparison), but lower sensitivity (ranging from 61.1% to 100% in the different comparisons).

The antibody L858R had 100% sensitivity in detecting L858R mutation on exon 21 and was completely negative in the case with the alternative point mutation L861Q+L862L on the same exon. The antibody E746-A750del was less sensitive, as it detected 6 of 9 cases with the specific mutation and 6 of 12 cases with all mutations in exon 19.

Previous studies with the same antibodies reported conflicting results (Table 6). Kawahara (140) described lower sensitivity for the anti E746-A750del antibody (75%), whereas Brevet (139) , Kato (141) and Kitamura (142) found lower sensitivity for anti-L858R antibody (94%, 75%, and 32%, respectively). Overall, the sensitivity values obtained in the different studies are comparable to ours. Nevertheless, Kitamura et al reported 32% sensitivity for L858R (142); this study has been performed using tissue microarrays (TMA), whereas we used whole sections, probably allowing us a more complete evaluation of the tumoral immunoreactivity. In our series, immunoreactivity was sometimes variable in different neoplastic areas; therefore, use of whole sections is advisable to avoid false negative results. However, we did not consider patchy staining and, in positive cases, immunoreactivity was always more than 70%. All previous studies are concordant in showing higher specificity (from 92% to 100%) than sensitivity (from 39% to 100%) for both antibodies (139-144). Similarly, in our series we described very high specificity (100% in each comparison), but lower sensitivity (ranging from 61.1% to 100% in the different comparisons).

In determining EGFR immunoreactivity, one of the crucial points was to differentiate score 2+ from 1+, because 2+ was considered positive for mutational status and 1+ negative, as

previously suggested by Kawahara et al. (140). However, the frequency of 2+ staining is low as it was detected only in one case with the antibody L858R and in one case with the antibody E746-A750del. In these cases, 2+ and 1+ immunoreactivities could be reliably distinguished by staining intensity, percentage of positive cells and membrane reinforcement.

IHC has distinct advantages over standard sequencing methods. First of all, it is less expensive and is more widely available. Secondly, IHC is a rapid procedure and time is critical in treating advanced pulmonary neoplasms. Thirdly, IHC may provide reliable results even on limited amount of material, i.e. small biopsies or cytological samples. Kawahara et al. (145), have reported 100% sensitivity and 100% specificity in a series of 24 patients with cytological samples composed of pleural effusions and cerebrospinal fluids. Finally, IHC allows to detect the tissue distribution of the mutated cells. This might be useful to evaluate cases with combined histology and to improve correlation of mutational status with pathological features (8).

Considering the high specificity of the test, IHC may be used for up-front selection of patients which could benefit from TKI therapy, reserving DNA sequencing for negative and/or suspicious cases. A similar strategy is currently applied in breast carcinomas for Cerb-B2 testing. Cerb-B2 is initially evaluated by IHC that can provide negative, positive or ambiguous results; in the latter case (score 2+) further molecular studies, i.e. fluorescence in situ hybridization (FISH), are performed (2). Other mutation-specific antibodies are currently being evaluated for clinical use, such as antibodies detecting EML4-ALK gene fusion products (146).

In conclusion, mutation-specific EGFR antibodies are sufficiently accurate to be used in routine practice to perform a first-line screening of patients candidate to TKI-therapy, as they are less expensive and time-consuming than traditional DNA sequencing. DNA sequencing analyses should be always performed in negative or suspicious cases.

Ref. (N.)	N. of cases studied	IHC methodology	Genetic/molecular test used	IHC scoring criteria	Sensitivity in detecting EGFR mutations			
					E746-A750del antibody in identifying the specific mutation	*E746-A750del antibody in identifying mutations of exon 19*	*L858R antibody in identifying the specific mutation*	*L858R antibody in identifying mutations of exon 19*
Brevet et al (139)	218	TMA	PCR-RFLP assays and sequencing for selected cases	4 grades, visual scoring	20/20 (100%)	17/35 (49%)	17/18 (94%)	NS
Kawahara et al (140)	45	Individual slides	Exons 19 and 21 sequencing	4 grades, visual scoring	9/12 (75%)	10/19 (53%)	15/19 (79%)	No alternative mutations
Kato et al (141)	70	TMA	Exons 18 to 21 sequencing	H score, cut off values at 20	9/11 (81.8%)	9/18 (50%)	9/12 (75%)	No alternative mutations
Kitamura et al (142)	238	TMA	Exons 19 and 21 sequencing	4 grades, digital scoring	NS	16/41 (39%)	NS	12/37 (32%)
Simonetti et al (143)	78	Individual slides	Exons 19 and 21 sequencing	4 grades, visual scoring	17/17 (100%)	17/29 (59%) 29 (69%)	25/25 (100%)	25/27 (93%)
Ilie et al (144)	61	TMA	Exons 19, 20 and 21 sequencing	4 grades, visual scoring	8/8 (100%)	8/9 (89%)	No mutations in exon 21	No mutations in exon 21
Current study	33	Individual slides	Exons 18, 19, 20 and 21 sequencing	4 grades, visual scoring	6/9 (66.7%)	6/12 (50%)	5/5 (100%)	5/6 (83.3%)

Table 6. Summary of all previous reported studies. *Legend*: TMA tissue micro array, RFLP restriction fragment length polymorphism, NS not specified.

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