

Fibroblast activating protein- α expression in squamous cell carcinoma of the esophagus in primary and irradiated tumors: the use of archival FFPE material for molecular techniques

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Abstract

There are numerous reports suggesting that fibroblast activating protein- α (FAP- α) plays an important role in invasion of various tumor types. We studied the expression pattern of FAP- α in esophageal squamous cell carcinoma (ESCC) patients who had not been treated primarily and those who had received neoadjuvant radiochemotherapy. Our goal was to establish whether readily available tissue specimens fixed in formalin and stored in paraffin blocks for years might still be a source of FAP- α RNA for PCR analysis. The study included 20 patients divided into two groups, 10 patients in each group. We evaluated the expression of FAP- α by PCR techniques in fresh frozen and in paraffin-embedded tissues, and compared it to the expression in non-cancer tissues. To detect the protein expression level of FAP- α in paraffin-embedded tissues we used chromogenic immunohistochemical (IHC) staining. Data were analyzed by *t*-test or the non-parametric Wilcoxon matched pair test using Statistica 12.5 software. We observed an increased level of the FAP- α gene and protein expression in cancer tissues when compared with their corresponding normal tissues. However, statistically significant differences were found only in the group of patients untreated before surgery. RNA extracted from paraffin-embedded tissue sections had very low quality, especially in the context of degradation. FAP- α remains a

highly altered participant of a complex microenvironment in esophageal squamous cell carcinoma, and its role in cell signaling requires further study. In this paper, we conclude that the use of a regular RT-PCR method for diagnostic purposes, which we have presented in an earlier paper, can be as good as qRT-PCR. Also, immunohistochemistry proved to be very useful and the only reliable method that can be used on long-term stored formalin-fixed, paraffin-embedded tissues.

Introduction

In recent years, attention has been paid to the close association between cancer and its microenvironment considering tumor growth, potential for invasion and metastasis. There are many reports suggesting that fibroblast activating protein- α (FAP- α) plays an important role in invasion of many tumor types. FAP- α is a serine protease that belongs to the family of transmembrane proteases, to which also dipeptidyl peptidase IV (DPPIV) belongs. They are grouped on the basis of their proteolytic activity, structure and behavior in their natural environment.¹⁻³ They share a unique ability to cleave the post proline bond that is usually resistant to degradation.⁴ FAP- α exists on the cell surface and in a soluble circulating form in the blood.^{5,6}

So far, little is known about the normal physiological role of either its cellular or circulating form. The cellular form of FAP- α possesses two lytic activities.^{7,8} It is best known for the endopeptidase activity. Gelatin is FAP's best explored substrate, and the gelatinase activity can be used to monitor its proteolytic activity. So FAP- α is involved in remodeling of extracellular matrix (ECM) with native and denatured collagens (gelatin). FAP- α is expressed as an active protease with no need for zymogen activation.⁹ The biological significance of FAP- α cleavage of gelatinase or collagen type I is still not known.^{10,11} Peptide hormones such as neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY are natural substrates for FAP- α dipeptidyl peptidase activity. The cleavage of peptide hormones involves FAP- α in influencing the tissue microenvironment.⁴

There is growing interest in FAP- α involvement in cell signaling. FAP- α is well known for its presence in stromal fibroblasts, called cancer-associated fibroblasts (CAFs), found in over 90% of epithelial tumors. However, tumor cells expressing FAP- α have been shown to possess either increased or decreased invasive behav-

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ior.^{12,13} Some studies have shown that FAP- α overexpression on tumor cells rather than on reactive fibroblasts was associated with more aggressive behavior of the tumor.¹⁴ Still, its expression is observed nearly exclusively in tumor stroma.⁹

Esophageal squamous cell carcinoma (ESCC) is a cancer with highly aggressive behavior and a 5-year survival rate of only 10%.¹⁵ The stroma associated with epithelial carcinomas, to which esophageal cancer belongs, remains a significant portion of its mass. It has been shown that the tumor is dependent on the reactive stroma for nutritional support, survival and progression. The stroma undergoes morphological alterations when attached to transformed epithelium: it recruits reactive fibroblasts, increases secretion of growth factors and proteases, and induces angiogenesis, to mention only a few activities.¹⁶⁻¹⁸ CAFs are essential to regulate the network of interactions occurring among the malignant epithelial cells, ECM and various noncancerous cells that are found in tumor surroundings, including endothelial, adipose, inflammatory and immune cells, and yet they remain an important part of the tumor milieu. FAP- α expression has been most extensively studied in CAFs associated with breast cancer and has relatively seldom been discussed in esophageal squamous cell carcinoma.¹⁹ Therefore, we studied the expression pat-

tern of FAP- α in esophageal squamous cell carcinoma patients who had not been treated primarily. We evaluated expression of FAP- α by immunohistochemistry and qRT-PCR in fresh frozen tissues and in paraffin-embedded tissues, and compared it to the expression in non-cancer tissue. Our goal was to establish whether readily available tissue specimens fixed in formalin and stored for years might still be used as a source not only for IHC staining but also for RNA analysis. We evaluated protein expression levels of FAP- α using immunohistochemistry, and the results were compared to data obtained by PCR techniques. Finally, we evaluated the FAP- α level alterations in a group of patients who underwent neoadjuvant chemoradiotherapy.

Materials and Methods

Patients and samples

The study included 20 patients (14 men and 6 women) divided into two groups, 10 patients in each group. Mean age of the patients was 59 (range: 45-74 years). All the patients were treated at the Department of Gastrointestinal and General Surgery of Wrocław Medical University (Poland) between 2006-2007 and 2015-2016 and had histologically confirmed primary ESCC. The first group included those patients who had received no previous treatment before surgery of the esophagus. In this group, all individuals had tumors diagnosed as pT2 or pT3, N0-N2 and M0 according to the TNM classification, and the second group had tumors diagnosed as pT2 or pT3, N0-N3 and M0 according to the pTNM classification (applied according to the guidelines from the American Joint Committee on Cancer staging manual) and received neoadjuvant chemoradiotherapy before surgery. As a result of the treatment, six of these patients (60%) were cancer negative on histopathological analysis after esophagectomy. None of the patients had any other serious diseases. The patients were informed about the potential use for research purposes of their surgically resected tissues. The study was approved by the ethics committee. The study was approved by the university committee for internal grants (grant number ST-879). The committee decided it did not require special authorization by the University Ethical Committee because our examinations were carried out in parallel with the histopathological examinations on the material that was used for standard procedures and had already been surgically removed for curative resection.

All the analyzed esophageal cancer specimens obtained during surgery were paired with nonmalignant tissue samples taken from the same individuals from the distal area of the esophagus, located 5 to 10 cm away from macroscopic tumor changes. Using histological examinations control samples were confirmed to be disease free (R0). Tissue samples were immediately put on ice, weighed, divided into pieces, snap-frozen in liquid nitrogen and kept at -80°C until further use. For immunohistochemical studies the tissue samples were fixed (for up to 7 days) in 10% formaldehyde solution in PBS (1:10 v/v) and then embedded in paraffin. RNA isolation from frozen tissues and formalin-fixed, paraffin-embedded tissue blocks, 30-50 mg fragments of frozen tissue were placed in a RLT lysis buffer (supplemented freshly with B-mercaptoethanol) supplied with the RNeasy Mini kit (Qiagen). Tissue was allowed to thaw, homogenized by scissors and processed further according to the manufacturer's instructions. RNA was eluted in 50 μL of RNase-free water. Total RNA from formalin-fixed, paraffin-embedded (FFPE) tissues was extracted using the RNeasy FFPE Kit (Qiagen). FFPE blocks were cut into sections and subjected to xylene/ethanol deparaffinization. Deparaffinized samples were incubated in lysis buffer supplemented with proteinase K (56°C for 15 min, then at 80°C for 15 min). During the isolation, genomic DNA was eliminated by DNase I treatment. RNA was eluted from the column in a volume of 30 μL of RNase-free water. Quantity and quality of obtained RNA were analyzed by NanoDrop measurements at OD of 260 nm, and A260/A280. Aliquoted total RNA samples were stored at -80°C until further use.

qRT-PCR

The cDNA used as a template for real-time PCR was produced using the SuperScript III First Strand Synthesis System for the RT-PCR Kit (Invitrogen by Life Technologies, Carlsbad, CA, USA). Total RNA samples (50 ng/mL) were reverse transcribed to cDNA according to the manufacturer's protocol. cDNA synthesis reactions were primed using random hexamers (10 min at 25°C , followed by 50 min at 50°C) and the reaction was terminated at 85°C for 5 min. After cDNA synthesis, the RNA template was removed by digestion with RNase H (20 min at 37°C). cDNA was stored at -80°C and then used in the PCR reaction (4 μL of cDNA mixture per 20 μL reaction). Quantitative real-time PCR primer pairs were purchased from Sino Biological Inc. (Beijing, China), designed

by using SBI's proprietary primer design algorithm. To avoid genomic DNA amplification, at least one primer crosses the junction of exons. GAPDH (glyceraldehyde-3-phosphate dehydrogenase), which was chosen as a reference gene, and FAP- α transcripts were detected in the samples by presence of 108-bp or 97-bp amplification products, respectively.

qRT-PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a reaction mixture containing SYBR Green I dye and gene-specific primer pairs at the final concentration 0.8 nM. Reactions were conducted on the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA); thermal cycling conditions were as follows: polymerase activation and DNA denaturation at 95°C , followed by 40 cycles of 2 s denaturation at 95°C , 20 s annealing at 60°C , and 20 s extension at 60°C . For each of the RNA extractions, PCR reactions were done in duplicate, and a negative control (water) was included in each run. Small, nonspecific primer-dimers in the negative control runs were obtained. FAP- α mRNA level was normalized to the arithmetic mean of the housekeeping gene level and analyzed using the $2^{-\Delta\Delta\text{CT}}$ method.²⁰

Immunohistochemistry

The 4-5 μm thick paraffin embedded sections were dewaxed in two changes of xylene (10 min each), hydrated in decreasing concentrations of ethanol (95%, 70%, and 50%) and washed in water. For antigen retrieval, incubation in 10 mM sodium citrate buffer pH 6.0, and heating in a water bath (97°C for 40 min) were performed. Tissue sections were immunostained with polyclonal sheep IgG anti FAP- α antibody (Human Fibroblast Activation Protein alpha/ FAP- α Antibody, cat. no. Q12884 from R&D) in 5 $\mu\text{g}/\text{mL}$ dilution. The primary antibody was applied overnight at 4°C . The sections were then counterstained with Mayer's hematoxylin. Omission of the primary antibody served as a negative control. Semi-quantitative digital image analysis to detect the intensity of FAP- α staining was performed using the ImageJ software. Stained specimens were viewed under a light microscope (Nikon ECLIPSE Ci), and five random areas per each section (patient) were captured as digital images (1296×972 pixels) with a digital camera (SPOT Idea) at 20x and 40x objective magnification (Nikon Plan 20x/0.4 and 40x/0.65). For quantitative purposes three randomly selected areas of stroma in each picture were analyzed with the histogram in grayscale from 0 (white) to 256 (black),

using ImageJ 1.50a software. The fold change value was calculated as the ratio of the difference between mean gray values obtained for control and cancer tissues.

Statistical analysis

The results are expressed as the mean (\pm SD) or the median (range). Data were analyzed by *t*-test or the nonparametric Wilcoxon matched pair test using Statistica 12.5 software. Values of $P < 0.05$ were considered statistically significant.

Results

Real-time PCR analysis of FAP- α using RNA extracted from fresh frozen tissues.

The results from individual patient data obtained by the qRT-PCR assays for the treated and untreated group are shown in Figure 1 A,B. qRT-PCR data for each sample are presented as relative gene expression calculated by the $2^{-\Delta\Delta CT}$ method.

We observed an increased level of FAP- α gene expression in cancer tissues when compared with their corresponding normal tissues in both groups. However, statistically significant differences were found only in the group of patients untreated before surgery. For these patients, the mean value of FAP- α overexpression was 2.3-fold higher in tumor versus control tissues (Table 1), FAP- α was overexpressed at least >2 -fold in 6 (60%) of all examined patients, and the highest overexpression was about 12-fold (Figure 1). In the group of patients who underwent neoadjuvant therapy before surgery changes were not statistically significant (Table 1).

Real-time PCR analysis of FAP- α using RNA extracted from formalin-fixed, paraffin-embedded tissues

To determine the usefulness of FFPE samples stored in the archives of Wrocław Medical University as readily accessible historical material for FAP- α level molecular analysis, we isolated total RNA from such available material and performed real-time PCR. The selected FFPE samples were

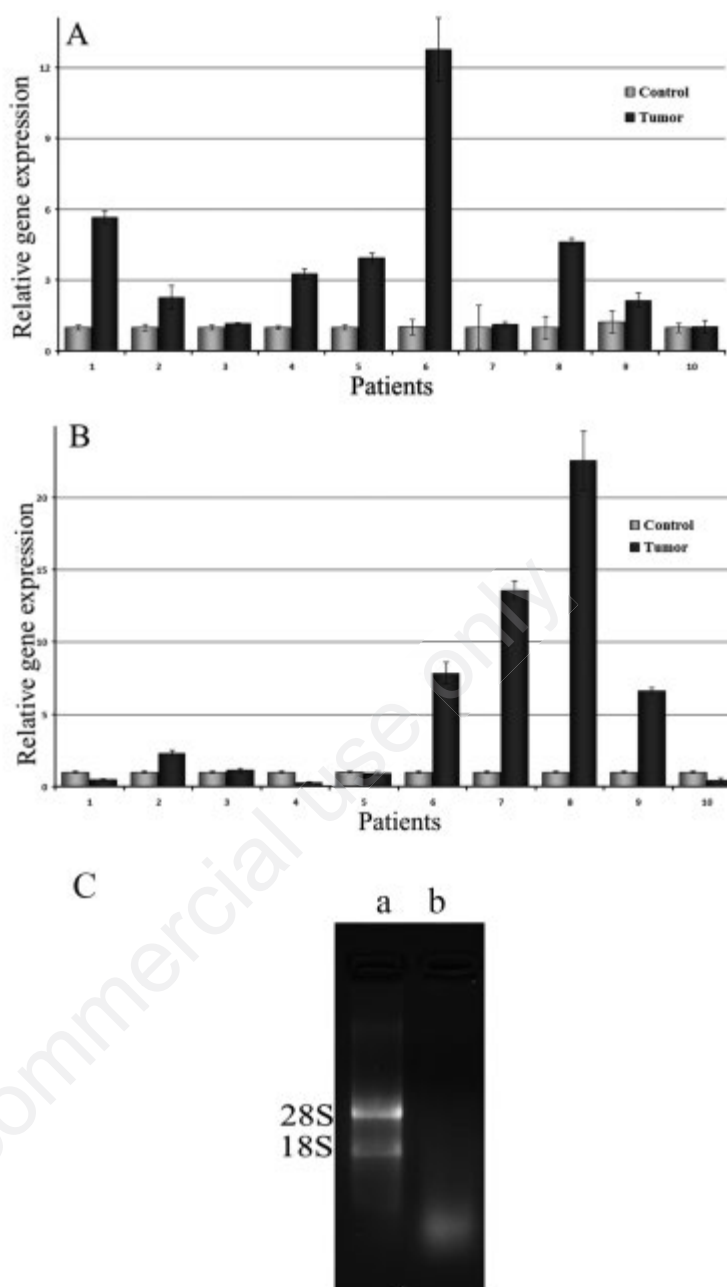


Figure 1. FAP- α gene expression level in ESCC tissues. Mean relative expression of FAP- α in fresh frozen esophageal squamous cell carcinoma tissues and nonmalignant tissues for patients (n=10) untreated (A) and patients (n=10) treated with chemoradiotherapy (B) before surgery, estimated by real-time PCR. C) Agarose gel of total RNA isolated from fresh tissue and FFPE sections. For fresh tissues the 18S and 28S ribosomal RNA bands are clearly visible (a), contrary to highly degraded RNA from FFPE, which appears as a low molecular weight smear (b).

Table 1. FAP- α gene expression fold change in ESCC patients with and without chemoradiotherapy before surgery, using qRT-PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, GAPDH mRNA expression as the reference gene and normal esophageal tissue as the calibrator. Data were analyzed using the nonparametric Wilcoxon matched pair test. Values of $P < 0.05$ are considered statistically significant.

Group	Fold change			Z	P-value
	Median	Minimum	Maximum		
Patients with no chemoradiotherapy before surgery, n=10	2.24631	1.03215	12.72255	3.01511	0.00256
Patients who received chemoradiotherapy before surgery (8-12 weeks), n=10	2.31965	0.07542	22.4737	0.55470	0.57910

ten years old, prepared according to the protocols routinely used in our hospital. To verify the procedure efficiency, we used RNA isolated from freshly collected tissue derived from the same patient as references. As could be expected, RNA extracted from paraffin-embedded tissue sections had a very low quality, especially in the context of degradation. On the agarose gel the RNA could be seen as a low molecular weight smear, contrary to RNA prepared from fresh tissues (Figure 1C).

For detection of FAP- α mRNA we used the standard real-time PCR reaction procedure which was used by us before. Regrettably, in regards of FFPE samples the results were not reliable, whereas for RNA isolated from fresh material and frozen for 10 years before usage, we obtained data which were fully compatible with calculations made previously by using reverse transcription PCR reactions.²¹

Immunohistochemistry

To verify whether changes at the mRNA stage reflect alterations at the protein level, we prepared the immunohistochemical staining of paraffin-embedded tissue sections from previously untreated patients with antibody directed against fibroblast activation protein. The obtained results were in accordance with the molecular biology data and showed an increase in protein level of FAP- α in tumor tissues when compared to marginal nonmalignant tissues taken from the same patient from the distal area of the esophagus characterized by the absence of macroscopic tumor changes. Histologically, FAP- α expression was restricted mostly to the stroma. The immunostaining with antibody in controls, cancer-free samples, was either very weak or locally moderate (Figure 2). Quantitative analysis of IHC staining confirmed a statistically significant difference between controls and cancer tissues. The average value of fold change was 2.7, where fold change indicates the cancer-to-control ratio (Figure 3).

Discussion

FAP- α evaluation is relatively new and has been used mostly for research purposes. Overexpression of serine proteases, to which FAP- α belongs, has been associated with cancer invasion and metastasis, essentially in gastrointestinal cancers.^{1, 22-24}

In the study, we evaluated FAP- α expression and protein distribution in the tumor mass in comparison to cancer-free samples in esophageal squamous cell carci-

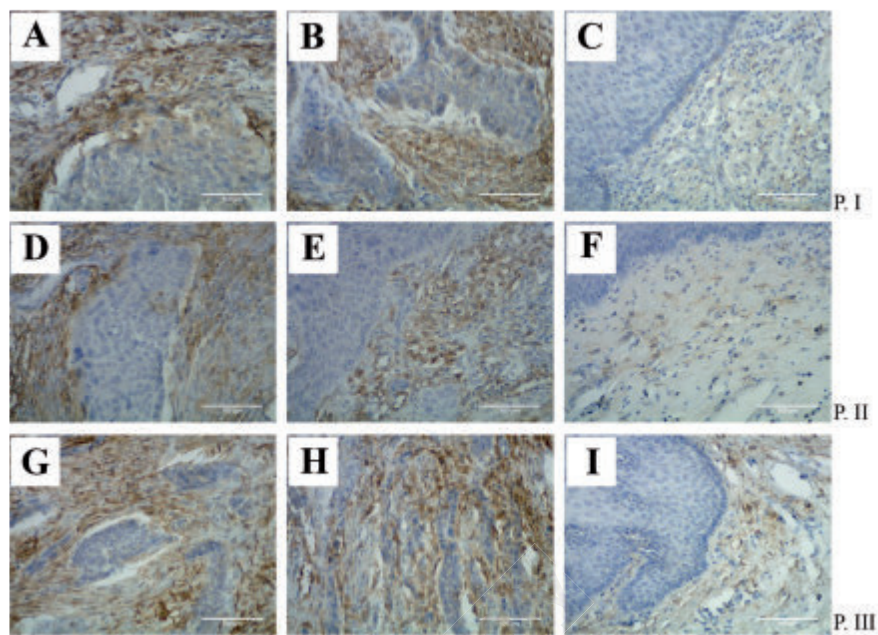


Figure 2. Examples of IHC staining patterns for FAP- α in the normal and tumorous tissues of the esophagus in three randomly chosen patients (P. I-III) untreated before surgery at a total magnification of 200x. Weak and locally moderate FAP- α immunoreactivity in normal epithelium (C, E, I). Strong FAP- α immunoreactivity in esophageal squamous cell carcinomas (A, B, D, E, G, H). Scale bars: 100 μ m.

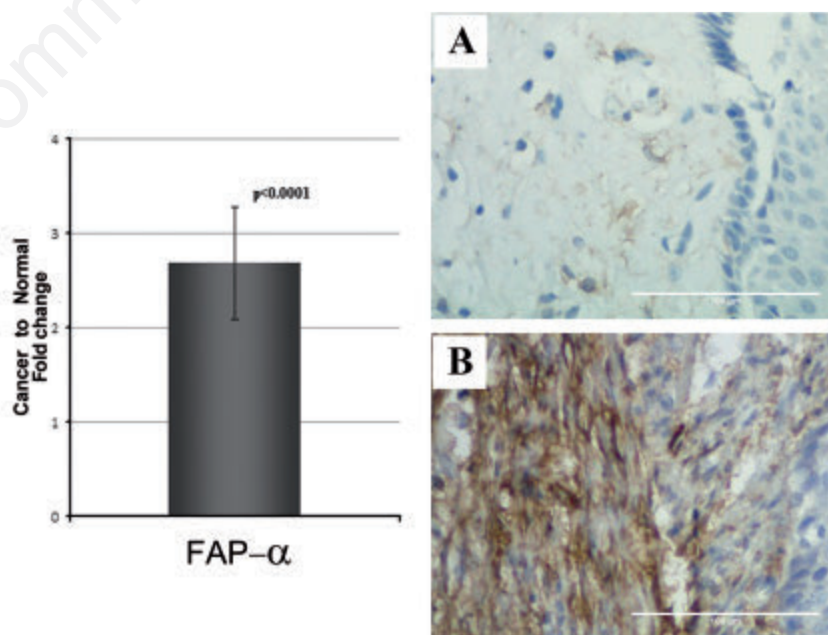


Figure 3. Quantification of IHC staining for FAP- α . Bar chart shows a statistically significant increase (average fold change of 2.7) in the intensity of staining in tumor tissues compared to normal tissues. The fold change indicates the ratio of mean gray values of normal to cancer tissues. Images are representative cases of IHC staining at a total magnification of 400x. They show the weakest (A) and the strongest (B) FAP- α immunoreactivity in esophageal normal and cancer tissues, respectively.

noma patients who underwent resection without preoperative treatment and after irradiation and chemotherapy. We also wanted to estimate the usefulness of FFPE samples stored in the archives of Wrocław Medical University as readily accessible historical material for molecular analysis of FAP- α level. We observed statistically significant overexpression of FAP- α as measured by both protein and RNA levels in the tumor tissue of the patients who did not undergo previous treatment ($P < 0.005$). However, we did not confirm these findings using real-time PCR methods on RNA extracted from formalin-fixed tissues, probably due to poor quality of the RNA we isolated from ten-year-old FFPE tissue specimens, fixed in 10% formalin. It is known that quality of the RNA extracted from paraffin-embedded tissue that undergoes chemical modifications and continues degradation over time can be low. The age and storage conditions including temperature and humidity of the tissue fixed in paraffin play a central role.²⁵ According to the published data, the longer the paraffin samples are stored, the more severe is the degradation observed. It seems that the critical storage time is 3 years.^{26,27}

FAP- α has been reported to be present in the tumor stroma of epithelial cancers including esophageal squamous cell carcinoma, essentially on CAFs, which contributed to tissue remodeling and invasiveness of the tumor by secreting proteases degrading the ECM and promoting the epithelial to mesenchymal transition.^{15,24,28-31} This is in agreement with our findings on all the samples of the tumor tissues in patients who did not receive any previous treatment. In these patients, the mean value of overexpression of FAP- α was 2.3-fold higher than in resections margins proved to be free from neoplastic cells on histology. However, in the group after previous chemoradiation, our findings were more interesting. FAP- α was generally more highly expressed in the tumor tissue than in the resection margins, but it was not as strong as in tumors with no previous treatment: 2.3-fold *versus* 2.2-fold stronger. The difference between the tumor tissue and normal tissue taken from the resection margin was not statistically significant for this group ($P > 0.005$). Even more important, FAP- α expression did not correlate with histological findings. In this group, almost all macroscopically abnormal tissues with the highest FAP- α level were cancer negative with negative lymph nodes on microscopic examination. Nevertheless, our observation is in agreement with Goscinski *et al.*, who noted a significantly decreased FAP- α

expression in irradiated patients.¹ FAP- α possesses collagenolytic activity which may increase during tissue remodeling processes: granulation tissue in healing wounds occurring in the esophagus after irradiation, the intensity of which is multifactor and requires further studies. A meta-analysis by Liu *et al.* showed that poorer prognosis among patients with epithelial tumors was significantly higher if FAP- α was overexpressed by tumor cells rather than fibroblasts.¹⁴ Our results may support the theory. Sixty per cent of the tumors were negative for cancer cells after chemoradiotherapy, so we conclude that FAP- α attached to cancer cells decreased in this group of patients, but was still present in granulation tissue when the irradiated site was healing. The hypothesis requires further verification with a larger patient cohort. It is known that FAP- α expression takes place not only in cancer tissues, but also in response to pathologic situations such as fibrosis and inflammation.³²⁻³⁵ Cancer development and wound healing are dynamic processes that have several similarities. This phenomenon is clearly in accordance with our results. The presence of high levels of FAP- α in tissues free from cancer after neoadjuvant therapy suggests its participation in extracellular matrix remodeling in both cancer and wound healing.

We evaluated FAP- α expression with PCR techniques on formalin-fixed, paraffin-embedded samples and on fresh-frozen tissues and attempted to validate these two RNA sources. We concluded that it is not possible to use our formalin-fixed, paraffin-embedded tissue to evaluate FAP- α alpha expression level using molecular techniques. However, the qRT-PCR analysis confirmed data obtained by regular RT-PCR when we used RNA isolated from fresh-frozen tissues.²¹ This shows that the regular RT-PCR method for diagnostic purposes was as good as the qRT-PCR method. We also confirmed that IHC staining remains a useful and very reliable method. Moreover, it was the only method that could be used on the formalin-fixed, paraffin-embedded tissues stored long term. The results of immunohistochemistry as confirmed by molecular methods are still the most important source of information on fresh and formalin-fixed tissues.

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