

PAPER DETAILS

TITLE: Evaluation of epidermal growth factor receptor in odontogenic tumors and rare soft tissue tumors by immunohistochemical and fluorescence in situ hybridization methods

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ARAŞTIRMA/RESEARCH

Evaluation of epidermal growth factor receptor in odontogenic tumors and rare soft tissue tumors by immunohistochemical and fluorescence in situ hybridization methods

Yumuşak dokunun nadir görülen tümörlerinde ve odontojenik tümörlerde immünohistokimyasal yöntem ve floresan in situ hibridizasyonla epidermal growth faktör reseptör varlığının gösterilmesi

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Abstract

Purpose: The aim of this study is to investigate the role of epidermal growth factor receptor (EGFR) in odontogenic tumors and rare soft tissue tumors.

Material and Methods: This study includes 28 cases (11 cases of odontogenic tumors and 17 cases of soft tissue tumors) which were diagnosed at Cukurova University Medical Faculty, Department of Pathology between 2002 and 2010. They were evaluated for immunohistochemical staining of EGFR and the presence of genetic abnormality by FISH method.

Results: Eleven cases were positively stained by immunohistochemistry. Eight cases had genetic abnormality of EGFR gene by FISH method. Nine of eleven odontogenic tumors (81,8%) showed overexpression of EGFR by immunohistochemical method or FISH method.

Conclusion: Our findings highlight that EGFR plays an important role in chordoma pathogenesis in soft tissue tumors. In addition to odontogenic tumors; two cases of vascular neoplasia, composite and retiform hemangioendothelioma, exhibited EGFR gene amplification. Interestingly, there is no available data about EGFR gene status in these tumors. EGFR can be used for diagnostic purposes. Also, it is a target for anti-EGFR therapy. Therefore, EGFR gene status is a valuable marker for therapeutic management.

Key words: Odontogenic tumor, Soft tissue tumor, EGFR, Immunohistochemistry, FISH.

Öz

Amaç: Bu çalışmada amaç, çeşitli odontojenik tümörler ve nadir görülen yumuşak doku tümörlerinde epidermal growth faktör reseptörün (EGFR) rolünü ortaya koyabilmektir.

Gereç ve Yöntem: Bu çalışmada 2002-2010 yılları arasında Çukurova Üniversitesi Tıp Fakültesi Patoloji Anabilim Dalı'nda tanı almış onbir odontojenik tümör olgusu ve onyediy nadir görülen yumuşak doku tümörü olgusu olmak üzere toplam 28 olgu incelenmiştir. Bu olgularda immünohistokimyasal olarak EGFR boyanması ve FISH yöntemi ile genetik anormallik araştırılmıştır.

Bulgular: Çalışmamızda tüm olgulara immünohistokimyasal olarak uygulanan EGFR ile onbir olgu pozitif boyanmıştır. FISH yöntemi ile ise sekiz olguda genetik anormallik saptanmıştır. Odontojenik tümör olgularımızın dokuzunda (%81,8) immünohistokimyasal yöntemle ya da FISH metodu ile EGFR overekspresyonunun saptanmıştır.

Sonuç: Yumuşak doku tümörlerinde ise kordoma patogeneğinde EGFR'nin önemi bulgularımızla tekrar vurgulanmış olup, daha önce EGFR varlığı gösterilmemiş olan odontojenik tümörler ile retiform ve kompozit hemanjoendotelyoma grubu tümörlerde de EGFR'nin rolü olduğu ileri sürülebilir. EGFR tanılarda amaçlı kullanılabileceği gibi, anti-EGFR ilaçların hedefini teşkil ettiğinden, hastaların tedavi yönetiminde de oldukça değerlidir.

Anahtar kelimeler: Odontojenik tümör, Yumuşak doku tümörü, EGFR, FISH, İmmünohistokimya.

INTRODUCTION

Odontogenic tumors and tumor-like lesions represent a heterogeneous group ranging from hamartomatous lesions to malign neoplasms. These lesions which are observed considerably rarely originate from epithelial, ectomesenchymal and/or mesenchymal tissues which try to form teeth. Malignant mesenchymal tumors are observed with a rate of 1% among all malignant tumors in humans¹. These tumors are important, since they are life threatening and prognosis and treatment is different for different subtypes, though they occur rarely. The Epidermal Growth Factor Receptor (HER1/EGFR) is a cell membrane receptor and its intrinsic protein has tyrosine kinase activity. EGFR is a member of erbB receptor family. In presence of Epidermal Growth Factor (EGF) or Transforming Growth Factor- α (TGF- α) ligands and with tyrosine kinase activation, HER1/EGFR combines with the other members of this family in such a way as to form hetero or homodimers. Thus, the receptor initiates a signal pathway which also involves RAS and mitogen activated protein kinase (MAPK). This pathway enables the cellular cycle to switch from G1 to S phase by affecting the nuclear proteins including cyclin D1^{2,3}.

Currently, the correlation of EGFR amplification with aggressive biological behavior and poor prognosis is well defined⁴. As a result of this, use of anti-EGFR antibodies affecting the signal pathway targeting EGFR ("targeted cancer treatment") is applied in treatment of different cancer types. However, EGFR has not been investigated in rare tumors of soft tissue and in a large portion of odontogenic tumors and therefore anti-EGFR modalities have not been tried in treatment.

The aim of this study was to investigate the state of EGFR which is a proto-oncogene in the above-mentioned tumor groups using both immunohistochemistry and FISH method and contribute to the pathogenesis, prognosis and treatment in these cases.

MATERIAL AND METHOD

Study Population

Twenty eight subjects who were diagnosed in Çukurova University, Medical Faculty, Department of Pathology between 2002 and 2010 were selected

for the study. 11 of these subjects had odontogenic tumor and the distribution was as follows: 1 odontogenic fibroma, 2 adenomatoid odontogenic tumors, 1 calcified epithelial odontogenic tumor, 1 compound odontoma, 1 ameloblastic fibroodontoma, 5 ameloblastomas. 17 had rare soft tissue tumor and the distribution was as follows: 4 chordomas, 2 epithelioid hemangioendotheliomas (HE), 1 composite HE; 1 retiform HE, 1 spindle cell hemangioma, 1 glomus tumor, 4 clear cell sarcomas, 3 low grade fibromyxoid sarcomas (LGFMS). The paraffin-embedded blocks and slides were taken out of the archives, reviewed and the most appropriate blocks were selected for immunohistochemistry and FISH methods. EGFR was studied in all subjects using the immunohistochemistry method and FISH method.

Immunohistochemistry

Four micron sections from the blocks selected for application of EGFR primary antibody with immunohistochemistry method were taken to polylysine slides (PLL). They were treated with dry and heat air for dehydration. They were kept at 0°C for 5 minutes for adhesion of the tissues. Afterwards, they were treated with dry and heat air again, kept in warm xylene in 56°C incubator for deparaffinization process for 10 minutes and kept in

Three consecutive xylene sets for one minute each. They were kept in 3 distilled water sets for one minute each to remove ethanol. The sections were washed with distilled water after being kept in 3% H₂O₂ solution for 5 minutes to suppress the activity of endogenous peroxidase in the tissue. The sections were placed in a reserved plastic chalet which contained citrate (pH 6) and were applied microwave process for 20 minutes at a moderate level. They were let to cool in a reserved way for 45 minutes. Afterwards, they were washed with distilled water and three separate phosphate buffer solutions (PBS) and EGFR antibody was dropped on the tissues. Following 60-minute incubation, the sections were washed in 3 separate PBS solutions and were kept for 20 minutes after Biotinylated Link Universal was dropped on top. Following washing with PBS again, 2-3 drops of streptavidin peroxidase solution were dropped and kept for 20 minutes. After the sections were washed with PBS again, they were kept in AEC chromogene for 10 minutes to enable visualization of staining. After staining with PBS opposite staining with haemotoxyline was

performed for 5 minutes. Subsequently, the sections were washed with tap water and closed with water-based material.

Membraneous EGFR was considered specific and scoring was made in two ways⁵:

1. According to the percentage of staining of the tumor cells;
 - No staining
 - Staining of less than 10% of the tumor cells
 - Staining of 10-50% of the tumor cells
 - Staining of more than 50% of the tumor cells
2. According to the intensity of membraneous staining in a single tumor cell;
 - No staining: negative
 - Weak, focal membraneous staining: (+)
 - Strong staining which does not fully surround the membrane: (++)
 - Strong and complete staining: (+++)

FISH Method

The sections prepared for the FISH method were kept in incubator at 56°C for one night. On the next day, 15 cc distilled water and 150 microliter 1 mole HCL were added in a separate slide box for each 5 slides and placed in a beaker filled with water. This beaker was put in an incubator with a temperature of 37°C. The water bath was adjusted to 80°C and deparaffinization prewashing solution was placed in a heat-proof reserved chalet. The slides which were taken out of the incubator were kept in three separate chalets containing xylene for 10 minutes each. The slides were dried at room temperature. The dried slides were kept in the deparaffinization prewashing solution for 30 minutes. At the end of the 30-minute period, the slides were rinsed with distilled water for 10-15 seconds. Just before the slides were placed in the slide box, the enzyme reactive diluted with 150 microliter distilled water was added into the mixture of distilled water and 1 mole HCL inside the slide box which was previously kept in incubator at 37°C. The slides were kept in enzyme study solution in the slide box in the incubator with a temperature of 37°C for 15 minutes. At the end of the 15-minute period, the slides were rinsed with distilled water which was at room temperature for 10-15 seconds. The slides were kept in 2XSSC solution at room temperature for three minutes for two times consecutively. The slides which were processed with 70%, 85% and 100% alcohol series for three minutes each were

dried. After the deparaffinization stage was completed, the denaturation and probing procedure was started. In evaluation of the cases, all areas with tumor were reviewed and were evaluated as stated in the literature⁶.

- High-level amplification: presence of 10 or more signals
- Polysomy: presence of more than two matched signals in more than 50% of the tumor cells
- Low-level amplification: presence of 3-10 signals in more than 50% of the tumor cells without polysomy
- Minimal aberration: presence of 3-10 signals in less than 50% of the tumor cells
- Disomy: presence of dual signal in 80% of the tumor cells

This Project with protocol number TF2009LTP59 was approved by the Research Ethics Committte of Çukurova University Medical Faculty with the decision number 3 made at the 7th meeting on 04.14.2011.

Statistical Analysis

SPSS 16.0 for Windows program was used to evaluate the subjects. The categorical measurements were summarized as figures and percentages and the numerical measurements were summarized as mean and standard deviation.

RESULTS

Fifteen (53.6%) of the subjects included in the study were male and 13 (46.4%) were female. The age range was between 6 and 82 years old. The mean age was 43.2 and the median age was 44,5. EGFR was positive in 9 of 11 odontogenic tumor cases and negative in 2 by immunohistochemically. The odontogenic fibroma had more than 50% tumor cells were EGFR (+++) positively stained. One of adenomatoid odontogenic tumor had EGFR (++) staining more than 50% of the tumor cells and the other adenomatoid odontogenic tumor had 10-50% of the tumor cells of EGFR (++) staining. More than 50% of the tumor cells were stained with EGFR (++) in calcified epithelial odontogenic tumor. Ameloblastic fibroodontoma had EGFR (+++) staining in more than 50% of the tumor cells, EGFR (++) staining in more than 50% of the tumor cells were found in three of 5 patients with ameloblastoma and cytoplasmic staining was found

in less than 10% of the tumor cells in one, whereas the other patients with ameloblastoma and compound odontoma were negative. Among 17 soft tissue tumor cases, EGFR (++) staining was found in more than 50% of the tumor cells in 2 of 4 chordomas, whereas the other two patients with chordoma, all 6 patients with vascular neoplasia, all 4 patients with clear cell sarcoma and all three patients with LGFMS were EGFR negative.

Among 20 patients in whom an adequate result was obtained with the FISH method, 6 patients showed

low-level amplification. Three cases were chordomas (Figure 1, Figure 2), One of these was ameloblastoma (Figure 3, Figure 4), one was retiform HE and one was composite HE. Minimal aberration was found in two patients including one patient with odontogenic fibroma and one patient with ameloblastoma. Disomy was present in 12 patients. Two of these patients had adenomatoid odontogenic tumor, one had calcified odontogenic tumor, one had chordoma, one had epitheloid HE, one had glomus tumor, two had LGFMS and four had clear cell sarcoma (Table 1).

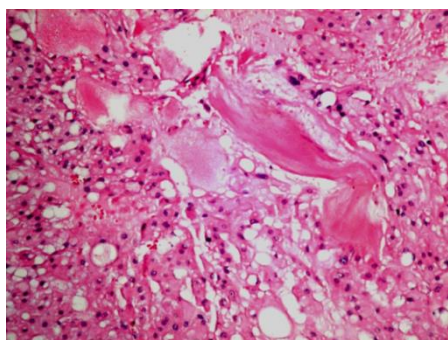


Figure 1a (H&E, X100): Pleomorphic atypical cells with markedly vacuolated cytoplasm, Chordoma

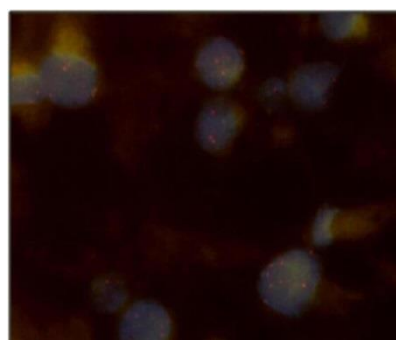


Figure 1b. Amplification in chordoma

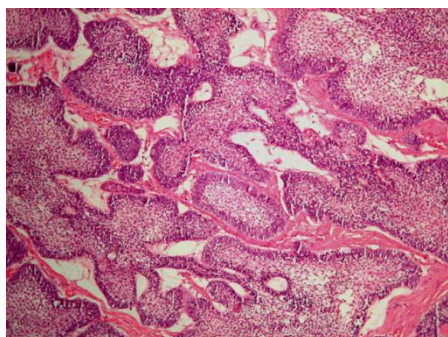


Figure 2a (H&E, X100): Peripheral palisading columnar basal cells with hyperchromatic nuclei, ameloblastoma

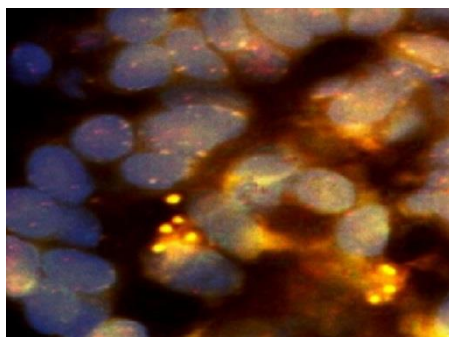


Figure 2b. Amplification in ameloblastoma

Evaluation could not be performed in 8 of 28 patients because of various causes. The tissue was shed in one patient with ameloblastoma and in one patient with epitheloid HE. Signal could not be detected in one patient with compound odontoma, two patients with ameloblastoma, one patient with ameloblastic fibroodontoma, one patient with spindle cell hemangioma and one patient with LGFMS. Low-level amplification was found in three of 11 patients who had EGFR positivity with

immunohistochemical method. Minimal aberration was found in two patients, disomy was found in three patients, signal could not be detected in two patients and evaluation could not be made in one patient, since the tissue was shed. Among 17 patients in whom the immunohistochemical method showed a negative result, low-level amplification was found in three, disomy was found in nine and evaluation could not be made in four, since the tissue was shed (Table 2)

Table 1. Tumor type and FISH results

Tumor type	FISH					
	Disomy	Minimal aberration	Low-level amplification	Polysomy	No Signal	Shed
Odontogenic fibroma		1				
Adenomatoid odontogenic tumor	2					
Calcifying epithelial odontogenic tumor	1					
Compound odontoma					1	
Ameloblastoma		1	1		2	1
Ameloblastic fibroodontoma					1	
Spindle cell hemangioma					1	
Epitheloid HE	1					1
Glomus tumor	1					
Retiform HE			1			
Composite HE			1			
Clear cell sarcoma	4					
LGFMT	2				1	

Table 2. .EGFR IHC (intensity) and FISH results

IHC	FISH				
	Disomy	Minimal Aberration	Low-level amplification	No Signal	Shed
Negative	9	0	3	4	1
(+)	0	0	0	0	1
(++)	3	1	3	1	0
(+++)	0	1	0	1	0

DISCUSSION

EGFR has an important role in development of normal tissue and progression of human neoplasms as other growth factors and receptors. One of the first studies related with EGFR was the study of Davies et al. which showed the localization on chromosome 7⁷. Veale et al. immunohistochemically showed EGFR positivity in non-small cell carcinomas of the lung⁸. EGFR has also been investigated in breast cancer, malign gliomas, salivary gland tumors, gastric carcinomas and bladder tumors⁹⁻¹³. In the literature, EGFR has also been investigated in non-neoplastic/benign lesions. Normal kidney and neoplastic renal tissue, meningiomas, normal epithelium of the cervix and cervical intraepithelial neoplasias, severe dysplasias of the vocal cord, malignant lesions of the thyroid gland, toxic goitre lesions, benign and malignant lesions of the breast, normal superficial epithelium of the ovary, ovarian inclusion cysts and ovary tumors originating from the superficial epithelium have also been investigated in terms of EGFR¹⁴⁻²¹.

Shrestha et al. investigated EGFR expression in odontogenic cysts and tumors and found positivity in odontogenic cysts similar to the cellular membrane in the normal epithelium²². Odontogenic tumors did not show expression. In one study conducted by Vered et al., it was reported that EGFR was a receptor which normally had a role in the development of oral mucosa and odontogenesis²³. Clark et al. evaluated expression of p 53, Ki 67 and EGFR before and after excision in odontogenic keratocysts which have a typical characteristic of demonstrating recurrence²⁴. In this study, increased EGFR expression was found in 13 of 16 patients before excision and increased to 15 after excision. This was interpreted as tendency of odontogenic keratocysts to EGFR-related proliferation.

In our study, there was 11 patients with odontogenic tumors and 9 of these patients had a positive stained EGFR by immunohistochemical method. Three of four patients with ameloblastoma whom showed EGFR (++) cytoplasmic staining in more than 50% of the tumor cells and one had EGFR (+)

cytoplasmic staining in less than 20% of the tumor cells. Low amplification was found in one of five patients with ameloblastoma with the FISH method and minimal aberration was found in one. Both patients showed EGFR (++) staining immunohistochemically.

EGFR expression has been demonstrated in ameloblastomas in some studies²³⁻²⁸. Among epithelial odontogenic tumors, ameloblastoma is the one which shows strong EGFR expression²⁹. It is known that ameloblastomas are radioresistant tumors. Therefore, ameloblastomas which show local aggressive behavior are appropriate candidates for anti-EGFR treatment. The preparation phase is very important in determining EGFR expression with immunohistochemical method in ameloblastomas. In our study, one patient with ameloblastoma lacked EGFR staining and no signal could be detected with the FISH method. In one patient with ameloblastoma who was referred from an external center for consultation, EGFR (++) staining immunohistochemically in more than 50% of the tumor cells and no signal was detected with the FISH method. The preparation phase is a multi-step process and a change in one of these steps may lead to lack of immunohistochemical response and/or lack of response with the molecular method^{25,27,28}.

In the study of Oliveira et al. and in some other studies, the response of the cell to the proliferation stimulus increased in cases where EGFR was localized only in the cellular membrane, whereas a slower response occurred in cases where EGFR was localized only in the cytoplasm (internalized and inactive)³⁰⁻³³. In our study, (+) cytoplasmic staining was found in less than 10% of the tumor cells in one patient with ameloblastoma. In this patient, no recurrence occurred in 9 years. Recurrence was observed in two years in two patients with ameloblastoma who had (++) membranous staining with EGFR. Based on all these cases, it was concluded that EGFR constituted a very significant step in the pathogenesis in development of odontogenic tumors.

One of the limitations of this study is the odontogenic tumors are highly variable, there are examples from the all 3 WHO benign tumor groups, consequently, the small number of individual cases are not comparable. Further studies are needed for specific subtypes of odontogenic tumors.

EGFR has been studied in soft tissue tumors as well as in many studies and many tumor groups. Yang et al. investigated EGFR overexpression in a heterogeneous group of sarcomas³⁴. Swisher et al. reported that EGFR was intensified especially in the sarcomatous areas in uterine sarcomas and adenocarcinomas³⁵. EGFR overexpression has also been found in synovial sarcoma in different studies^{36,37}.

In the present study, EGFR was found to be positive with immunohistochemical method in two patients with chordoma among 17 cases of soft tissue tumor. 15 patients were negative. Low-level amplification was found with the FISH method in these two chordomas and in another chordoma who was immunohistochemically negative. The remaining patient with chordoma had disomy with FISH. In recent years, many studies showing EGFR expression have been conducted in relation with chordomas^{5,6,38-43}. Based on the findings and previous studies, it was concluded that immunohistochemistry alone was not a good marker in demonstrating mutation or copy number^{44,45}. In our study, EGFR overexpression was found by immunohistochemistry and/or FISH method in three of four patients with chordoma. One patient with chordoma was found to be EGFR negative immunohistochemically and disomy by FISH. The role of EGFR in the pathogenesis was emphasized with the findings of our patients with chordoma as stated in the literature.

In our study, there were four patients with clear cell sarcoma and these patients were found to be EGFR negative immunohistochemically and disomy by FISH. In some studies in which mRNA expression profile analysis was made in clear cell sarcoma, *erbB3* overexpression was found⁴⁶⁻⁴⁸. In another study, increased *erbB3* expression was found in clear cell sarcoma of 8 soft tissues and overexpression was not found in any of them⁴⁹. In the study presented, it can be predicted that EGFR does not play a role in the pathogenesis of clear cell sarcoma with the findings obtained in accordance with the literature.

The composite HE and retiform HE were EGFR negative immunohistochemically and had low-level amplification by FISH in this study. There is no such example in the literature. Our study had a small sample size. Thus, the importance of EGFR in the pathogenesis and treatment in these two tumor groups should be investigated in larger series.

One of limitations of our study is a non-optimal tissue processing for molecular testing. For instance, there were 8 patients three of whom were found to be positive EGFR staining by immunohistochemically and five of them lack EGFR. FISH material was not sufficient in these 8 patients. In two of them, the tissue was shed. One of these patients had ameloblastoma and the sample belonged to 2002 and was exposed to decalcification process. The other patient had epitheloid HE. Signal could not be obtained in the remaining 6 patients. Immunohistochemical EGFR positivity was present with varying rates in two of these patients. In the literature, there are many studies investigating the FISH technique and its limitations. In some of these studies, 1p and 19q probes of different trademarks were evaluated comparatively with PCR and FISH in patients with glioma and it was noted that weak signal was obtained with some probes⁵⁰⁻⁵⁴.

The main disadvantage of the FISH method is nuclear cessation artifacts and hybridization defect⁵⁵. Sufficient number of cells should be evaluated manually to obtain a statistically significant result. Therefore, it takes considerable time to evaluate and report this method. Aneuploidy and polyploidy signal counting may be confusing^{55,56}.

The FISH technique has three basic phases. Although fixation which is the first phase changes by the material to be used, some of the cases in our study were constituted of paraffine tissues fixed with formaldehyde exposed to decalcification process. Presence of FISH signal in tissues embedded in paraffine is evaluated with much more difficulty compared to conventional cytogenetic materials⁵⁷. The main problems include unsuccessful hybridization, resulting weak binding of probe, excessive probe need, background staining, autofluorescence and dividing in the nucleus⁵⁸⁻⁶¹.

It has been found that the findings obtained by immunohistochemical method and FISH method do not necessarily overlap. Although there are studies in the literature showing that the FISH method is specific than the immunohistochemical method, trouble in any phase in the preparation period or in the tissue follow-up period makes it difficult to make a healthy evaluation, since this method (FISH) has sensitive steps. Therefore, the tissue to be studied genetically should be prepared under optimal conditions.

It has been demonstrated that targeted therapies inhibiting EGFR-mediated pathways may also be beneficial in some soft tissue sarcomas⁶²⁻⁶⁵. However, large-scale, multi-center studies should be conducted to develop treatment modalities which would provide clinically significant benefit, since soft tissue sarcomas are observed rarely.

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