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EGFR Expression and Gene Copy Number in Malignant Pleural Mesothelioma in Turkey

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ABSTRACT

Malignant pleural mesothelioma (MPM) is an aggressive tumor with poor prognosis. Standart chemotherapy regimens still remain palliative. There is urgent need for novel therapy options such as targeted therapies in order to improve the outcome of the patients. Epidermal growth factor receptor (EGFR) is a common target for cancer chemotherapy. There is relatively less data in the literature regarding EGFR expression status in MPMs. In this study we analysed EGFR expression immunohistochemically in 21 Turkish MPM cases. As gene amplificaiton is one of the mechanism responsible from receptor overexpression, we also studied EGFR gene copy number by fluorescent in situ hybridization (FISH) method. We found EGFR overexpression in 16/19 (84.2%) cases. EGFR overexpression was seen in all epithelial and most (66,6%) biphasic tumor types. There was, however, no association between EGFR expression and survival. Although we did not find gene amplification, we detected gene copy number increase (e.g., high polysomy) in two cases (9,5%). Gene copy number increase seems to be responsible from EGFR overexpression in only a small percentage of MPM cases. Further studies investigating other potential factors which might be associated with EGFR expression are needed, and more clinical trials are required to evaluate whether MPM patients are candidates for EGFR targeting therapies.

Keywords: EGFR, Mesothelioma, FISH, Gene Copy Number, Immunohistochemistry

ÖZET

Türkiyede malign Plevral Mezotelyomalarda EGFR Ekspresyonu ve Gen Kopya Sayısı

Malign plevral mezotleyoma (MPM) kötü prognoza sahip agresif bir neoplazidir. Standart kemoterapötik ajanlar hala palyatif yarar sağlamaktadır. Hasta sağkalımının iyileştirilmesi için hedefe yönelik tedavi seçenekleri gibi yeni yöntemlere acilen ihtiyaç duyulmaktadır. Epidermal büyüme faktörü reseptörü (EGFR) kanser kemoterapisinde potansiyel bir hedeftir. Literatürde MPM'lerde EGFR ekspresyonu ile ilgili veri nispeten azdır. Biz bu çalışmada 21 Türk MPM olgusunda immünhistokimyasal olarak EGFR ekspresyonunu araştırdık. Reseptör aşırı ekspresyonlarından sorumlu mekanizmalardan birisi de gen amplifikasyonu olduğundan, floresan in situ hibridizasyon (FISH) yöntemi ile EGFR gen kopya sayısını sorguladık. 19 olgunun 16'sında (%84,5) EGFR aşırı ekspresyonu belirledik. EGFR aşırı ekspresyonunu epitelioid tümör tiplerinin hepsinde, bifazik tiplerin çoğunda (%66,6) gözlemledik. Ancak, EGFR aşırı ekspresyonu ve sağkalım arasında istatistiksel olarak anlamlı bir fark bulamadık. Gen amplifikasyonu saptamamakla birlikte, iki olguda (%9,5) gen kopya sayısında artış (yüksek polizomi) belirledik. Gen kopya sayısı artışı MPM olgularının ancak küçük bir yüzdesinde reseptör aşırı ekspresyonundan sorumlu gibi durmaktadır. EGFR aşırı ekspresyonundan sorumlu diğer potansiyel faktörleri araştıran başka çalışmalara ve MPM olgularında EGFR'yi hedef alan tedavilere yanıtı değerlendiren klinik araştırmalara ihtiyaç vardır.

Anahtar Kelimeler: EGFR, mezotelyoma, FISH, Gen kopya sayısı, İmmünhistokimya

INTRODUCTION

Malignant pleural mesothelioma (MPM) is an aggressive tumor causally related to asbestos inhalation, mainly with occupational exposure. SV40 exposure and genetic predisposition are also considered in the pathogenesis of some MPM cases.[1,2] In Turkey, MPM is an important health problem as it is endemic in certain regions such as Cappadocia and Southeastern regions due to environmental exposure to asbestos and also erionite, a fibrous zeolite.[3-7] MPM patients are rarely diagnosed at early stages when they may benefit from combination of surgery, radiotherapy and chemotherapy. However, systemic therapy, such as combination of pemetrexed (a folate anti-metabolite) and cisplatin which is considered to be the standart regimen, is the only therapy option for majority of cases as their tumors are unresectable due to advanced disease. Although the response rate is better with combination therapy than with single agents, overall survival of MPM patients is poor.[8] Better understanding the complex biology of MPM and development of new therapeutic agents against potential molecular targets may improve the prognosis of the disease.

Epidermal growth factor receptor (EGFR), a potential molecular target in cancer therapy, is a tyrosine kinase receptor which belongs to ErbB family. EGFR (also known as ErbB1/HER1) is broadly expressed in various epithelial and mesenchymal cells, and is involved in cell proliferation, inhibition of apoptosis, cell motility, angiogenesis and expression of extracellular matrix proteins.[9,10] Overexpression of EGFR has been reported in various neoplasms including non-small cell lung cancer (NSCLC), head and neck, breast, gastic, colorectal, ovarian and bladder cancers.[11,12] Since EGFR overexpression is associated with malignant phenotype in most tumors, several inhibition strategies, such as tyrosine kinase inhibitors (TKIs) including gefitinib and erlotinib have been developed and used in clinical practice.[13,14] EGFR overexpression has been shown between 32% and 100% of MPMs.[15-20] Some in vitro studies demonstrated that EGFR inhibition has been effective against MPM cell lines.[21-23] However, clinical studies with EGFR-TKIs, which are few in number, did not show significant improvement in the prognosis of EGFR-expressing mesothelioma patients.[24,25] These clinical studies, however, were based on immunohistochemical expression of EGFR. There may be several mechanisms responsible from EGFR overexpression, including gene amplification, mutation and/or reduced degradation of the protein.[11,26] Although immunohistochemical assessment of EGFR expression is a relatively easy and practical technique, it is not always predictor of response to TKIs. For example, trials of EGFR-TKIs in NSCLC showed a higher response rate in a subset of cases which show EGFR gene amplification or activating mutations in the tyrosine kinase domain of EGFR gene.[27-31] Despite a high rate of EGFR overepression in MPM, suggesting that it may play a role in the pathogenesis of the disease, it has been shown in a few studies that EGFR gene copy number increase and/or mutation is a rare event.[18,19,32,33] This may be an important issue in selecting candidates for EGFR-TKIs in MPM patients.

As far as we know, there is no report in the literature regarding EGFR status in Turkish MPM patients. In this study we aimed to investigate EGFR expression rate by immunohistochemistry and EGFR gene copy number as a possible underlying mechanism using fluorescent in situ hybridization (FISH) technique in 21 Turkish MPM patients. We correlated our findings with tumor histologic subtypes and patients survival.

MATERIALS AND METHODS

Cases: We analysed 21 malignant pleural mesothelioma cases which were diagnosed between 2001 and 2007 at Hacettepe University Faculty of Medicine Department of Pathology. All tumors were confirmed by immunohistochemistry using a panel of antibodies including calretinin, thrombomodulin, D2-40, WT-1, Glut-1, Ber-EP4 and CEA. A tissue microarray was constructed from tumor paraffin blocks.

Immunohistochemistry: EGFR expression was evaluated using immunohistochemical method (EGFR.25, 1:50, Novocastra). After 4-μm sections were made, the slides were deparaffinized and immunohistochemistry was performed with streptavidin-biotin-peroxidase method. We used a scoring system for EGFR evaluating the intensity and the extent of the positive membranous and/or cytoplasmic staining tumor cells as previously described in the literature.[18] The intensitiy of EGFR staining was scored as follows: 0 (absent), 1 (weak), and 2

(strong). Extent of the staining was evaluated according to the percentage of immunoreactive tumor cells as follows: 0 (0-10%), 1 (11-50%), and 2 (51-100%). We obtained three categories of EGFR immunoreactivity by multiplying the scores of the intensity and the extent of immunostaining as follows: negative (score 0): when there was no staining at all; low expression (score 1 and 2): when the staining was weak regardless of the extent of staining, or when there was strong staining in <50% of the cells; and high expression (score 4): when there was strong staining in >50% of the cells (2 1).

Fluorescent in situ hybridization (FISH) analysis: EGFR gene copy number in each tumor cell was assessed by FISH technique with the LSI EGFR SpectrumOrange/CEP7 SpectrumGreen probe (Vysis, Abbott Laboratories, IL, USA) according to protocol by Hirsch et al.29 Gene copy number was classified according to the number of EGFR genes and number of centromeric signals of chromosome 7 in each case, such as follows: 1) disomy (≤ 2 copies in >90% of cells); 2) low trisomy (≤ 2 copies in ≥ 40% of cells, 3 copies in 10% – 40% of the cells, ≥ 4 copies in <10% of cells); 3) high trisomy (≤ 2 copies in ≥ 40% of cells, 3 copies in ≥ 40% of cells, ≥ 4 copies in <10% of cells); 4) low polysomy (≥ 4 copies in 10% – 40% of cells); 5) high polysomy (≥ 4 copies in ≥ 40% of cells); and 6) gene amplification (defined by presence of tight EGFR gene clusters and a ratio of EGFR gene to chromosome of ≥ 2 or ≥ 15 copies of EGFR per cell in ≥ 10% of analyzed cells).[31]

Statistical Analysis: Statistical analysis was carried out using the SPSS 15.0 Software. Fisher's exact test was used to examine the correlation of immunohistochemical expression and gene copy number of EGFR detected by FISH. Statistical significance was set at $p< 0.05$. Kaplan-Meier test was used for survival analysis.

RESULTS

Cases: Surgical specimens included eight extrapleural pneumonectomy and thirteen video-assisted thoracic surgery (VATS) biopsy materials. Eleven tumors were located in left and ten in right side. Thirteen patients were male and eight were female. Age of patients ranged between 33 and 66 (mean 46,8). Tumor histologic subtypes included 14 epithelial (66,7%), 3 biphasic (14,3%) and 4 sarcomatous (19%). Patients with early stages were treated with surgery+radiochemotherapy and unresectable cases were treated with pemetrexed+cisplatin regimen. Patient follow-up was available in nineteen cases. Fourteen died of disease and remaining five were alive (mean 19.7 months, median14.0 months) at last evaluation. Patient data, tumor histologic subtypes, immunohistochemistry and FISH results are shown in Table.

EGFR expression: EGFR expression was evaluated by immunohistochemistry in 19 cases. Two cases with epithelial subtype were excluded from the evaluation due to technical problems in the cut sections. Membranous and/or cytoplasmic EGFR positivity was detected in 16 cases (84.2%) (Table). The immunoreactivity was strong in nine and weak in seven cases (Figures 1a and 1b). EGFR immunoexpression (when strong and weak stainings were accounted together) was seen in 12 of 12 epithelial, 3 of 3 biphasic and 1 of 4 sarcomatous mesotheliomas. All epithelial and biphasic tumors showed EGFR expression, while the rate of expression was 25% in those with sarcomatous histology ($p= 0.004$, Fisher's exact test). Among three cases from erionite region, two showed weak EGFR expression and one showed no expression. We did not find any correlation between EGFR expression and survival.

EGFR gene copy number: EGFR gene copy number in 21 malignat pleural mesothelioma cases were assessed using FISH technique. EGFR gene status was as follows: 2 high polisomy (9.5%) (Figure 2), 13 disomy, 6 low polisomy (Table). We did not detect any amplification in our mesothelioma series. We found that cases with increased EGFR gene copy number (e.g., two high polysomy cases) were restricted to epithelial tumor subtypes. These two FISH positive cases also showed strong EGFR immunoexpression. Among two FISH-positive cases, one was lost to follow-up, the other survived seven months. EGFR gene copy number of three cases from erionite region were as follows: one disomy and two low polysomy. We did not find any statistically significant correlation between EGFR expression, gene copy number, tumor type and survi-

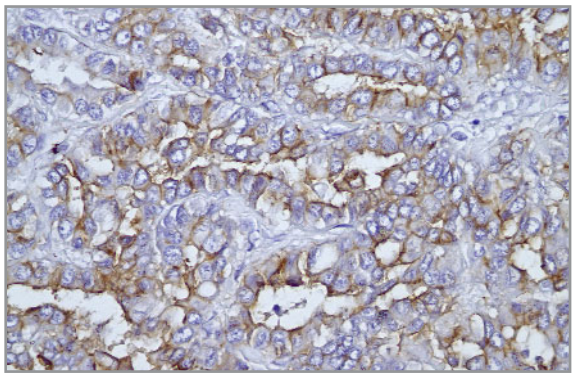

Figure 1a. An epithelial mesothelioma case showing strong and diffuse EGFR expression

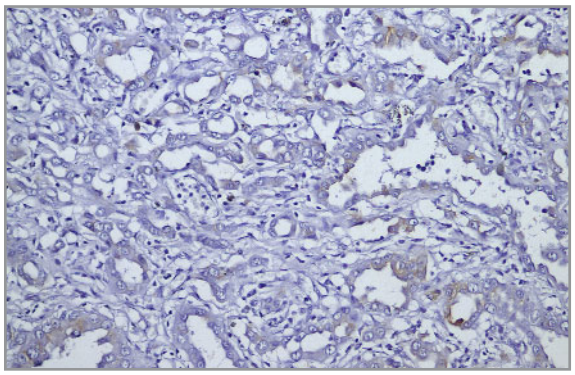

Figure 1b. EGFR expression is weak and focal in this epithelial mesothelioma case

val, which might be due to limited number of cases.

DISCUSSION

Malignant pleural mesothelioma is an aggressive neoplasm which mostly arises due to inhalation of asbestos fibers by occupational or environmental exposure. There is also some data suggesting that SV40 exposure and genetic predisposition might be associated with some cases.[1,2] In Turkey, it is an endemic disease because of environmental exposure to asbestos and erionite, a fibrous zeolite in certain regions of Turkey such as Cappadocian and Southeastern regions.[3-6] Majority of MPM cases present with advanced disease and are not candidates for surgery. Thus, pemetrexed, a folate anti-metabolite, in combination with cisplatin is considered the standart regimen in these unresectable cases. However, the overall prognosis of the disease is still poor.[8] As the pathogenesis of MPM is complex, there is no effective agent for treatment yet.

EGFR plays a central role in cell proliferation, differentiation, and survival.[9,10] It has also been shown to be overexpressed in various tumors including MPM. The rate of immunohistochemical expression of EGFR in MPM has been reported between 32% and 100% in the literature and those cases were mostly of epithelial tumor subtype.[15-20] Different antibody clones, methodologies and scoring systems may be responsible for this variation in expression. Although EGFR overexpression has been implicated as a poor prognostic feature in many solid tumors,[12] its relation with prognosis in MPM is not clear. Some authors did not find any difference in survival between EGFR positive and negative cases,[18,19] where others reported overexpression of EGFR was associated with favorable outcome.[15, 17] Recently, it has been claimed that EGFR immunoreactivity could be considered as a negative prognostic factor for MPM.[20]

In this study, we found EGFR expression in 16 of 19 cases (84.2%). All 12 (100%) epithelial and 2 of 3 (66.6%) biphasic tumor subtypes showed EGFR expression, where only 1 of 4 (25%) sarcomatous subtype demonstrated EGFR expression. The immunoreactivity was strong in nine cases and weak in seven cases. Although there seemed to be an association between EGFR expression and tumor histology (p= 0.004, Fisher's exact test), we did not show an association with patient survival, which might be due to the limited number of cases in the study. Overexpression of EGFR in many MPM cases suggests that it may contribute to the pathogenesis of at least some cases, and, thus those cases could potentialy benefit from tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib. Although in vitro studies with EGFR-TKIs have been reported to inhibit mesothelial proliferations, clinical trials did not show significant improvement in the prognosis of patients with EGFR-expressing tumors.[21,23-25] Presence of EGFR overexpression, however, is not always an indicator of TKI therapy, because underlying mechanisms such as gene amplification or mutation may be the predictor of therapy response as in the cases of NSCLCs. Trials of EGFR-TKIs in NSCLCs showed a higher response rate in a subset of cases which show EGFR gene amplification or activating mutations in the tyrosine kinase domain of EGFR gene.[27-31] In the literatu-

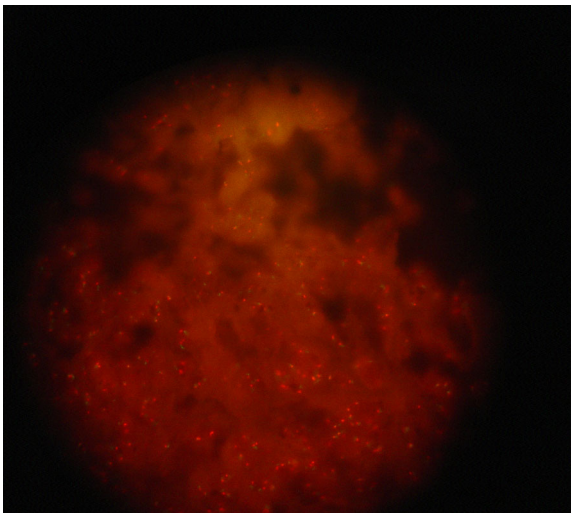

Figure 2. A mesothelioma case showing increased EGFR gene copy number (high polysomy) in FISH

re, there are only few mesothelioma series in which EGFR expression and mutation and/or amplification status have been evaluated.[18-20,33] Although the rate of EGFR overexpression is relatively high in MPM, it has been shown in these reports that EGFR gene copy number increase and/or mutation is a rare event. For example, Okuda et al. reported high polysomy in 4% of pleural mesothelioma cases with an overall EGFR expression rate of 32%. Rena et. al showed gene amplification in 8%, and EGFR expression in 46% of their mesothelioma series.[20] Recently, Enomoto et. al demonstrated only one high polysomy (3%) and EGFR expression rate of 53% in their study.33 When we analysed our 21 cases for EGFR gene copy number by FISH method, we found 2 high polysomy cases (9.5%). Those cases were limited to epithelial histology, which also showed strong EGFR expression immunohistochemically. We did not show gene amplification. Our findings were similar to those of the reported series in that, increased gene copy number seems to be responsible for EGFR overexpression in only a minority of MPM patients. In this study we did not study EGFR mutations. Although EGFR mutations are found in a subset of NSCLCs, most of the studies with EGFR-expressing pleural mesotheliomas did not show such mutations.[18,19] However, Enomoto et al. detected EGFR missense mutations in six out of 38 patients (16%) with pleural and peritoneal mesotheliomas. Foster et al. reported that EGFR mutation was found in 31% of cases of malignant peritoneal mesotheliomas.32 The low rate of EGFR gene copy number increase or abscence or/low rate of mutations might be a factor in poor response rates against anti-EGFR therapies in MPM patients. This subject merits further clinical and molecular studies.

In conclusion, according to our findings EGFR overexpression is a common feature in MPMs as shown by immunostaining in majority of our cases. Although EGFR expression was seen mainly in epithelial tumor subtype, which is known to be associated with favorable prognosis, the present study is far from claiming it to have a prognostic value. Gene copy number increase may be responsible from EGFR expression in only a minority of the cases. Further studies investigating other potential factors which might be associated with EGFR expression are needed, and more clinical trials are required to evaluate whether MPM patients are candidates for EGFR targeting therapies.

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