HIGH LIPOCALIN- 2 EXPRESSION INCREASES PEMETREXED SENSITIVITY IN PATIENTS WITH LUNG ADENOCARCINOMA

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ABSTRACT

Introduction*: The aim of this study is to determine the expression level of lipocalin-2 protein in human lung adenocarcinom tissues and to evaluate the relationship between its expression levels and clinicopathological parameters, including response to pemetrexed, degree of tumor differentiation, driver mutation status, progression free survival (PFS) and overall survival (OS).*

Materials and methods*: We retrospectively examined paraffin-embedded tissue sections from 30 metastatic lung adenocarcinoma patients whose clinical outcomes had been tracked after pemetrexed treatment. The expression status of Lipocalin-2 was determine by immunohistochemistry (IHC) using the anti-lipocalin-2 antibody.*

Result*s: Lipocalin-2 was highly expressed in 56% of the examined tumor tissues. There was significant association betwen high lipocalin-2 expression and increased pemetrexed sensitivity (p=0,028). There was no correlation between the degree of tumor differentiation and the level of lipocalin-2 expression. All five patient with EGFR mutation showed high lipocalin-2 expression (p=0,043). Kaplan-Meier survival analysis showed a significant correlation between expression levels of lipocalin-2 and PFS (p=0.014). Whereas there was no significant correlation betwen level of lipocalin-2 and OS although patients with high levels of this protein had a marginally longer survival time compared to those with low levels.*

Conclusion*: Our data suggest that lipocalin-2 may be a predictive marker for pemetrexed effectiveness in patients with lung adenocarcinoma.*

Keywords: *Cancer, lung, Lipocalin-2, pemetrexed, predictive, prognostic.*

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Introduction

Lung cancer is the most common cause of cancer and cancer-related deaths in men and the third most common cause of cancer and the second most common cause of cancer-related deaths in women globally[(1)]. Pathologically, it is divided into two categories as small cell and non-small cell lung cancer. Adenocarcinoma belongs to the group of non-small cell lung cancer and is the most common subtype of all lung cancers with a rate of more than 40%[(2)]. Despite the advances in treatment such as immunotherapy and targeted treatments, all patients with metastatic lung cancer show progression and die of their disease.

Pemetrexed is chemically similar to folic acid and is in the class of chemotherapy drugs named folate antimetabolites. It has more efficacy

in nonsquamous NSCLC than squamous NSCLC. Clinical trials have demonstrated 40% responses as a combination with platinium in first-line treatment and 10% responses as a single agent in sequent line treatment[3]. Pemetrexed plus platinum doublet are the best chemotheraupetic agents for lung adenocarcinoma that do not harbor driver EGFR mutation altought a proportion of patients do not benefit from cehmotherapy[3]. For this reason, determination of predictive and prognostic factors in lung cancer may be helpful to select those patients more likely to derive a clinical benefit.

Lipocalin-2, (also known as neutrophil gelatinase-associated lipocalin, oncogene 24p3 and uterocalin) is a glycosylated protein that was originally purified from neutrophil granules[4,5]. Lipocalin-2 function as transporter of some lipophilic molecules, such as retinoids, fatty acids, and cholesterol. Moreover, It is involved in iron delivery, cell migration, apoptosis, and cell differentiation. The best-characterised function of lipocalin-2 is to deprive bacteria of iron essential to their growth. Thus lipocalin-2 plays an important role in the innate immune response against bacterial infection[6,7]. Surprisingly, expression of lipocalin-2 has been shown to increase in many types of cancer in recent years, including pancreas[8], breast[9], ovarian[10], esophageal[11,12], gastric[13] and lung cancer[14]. It increases activity of matrix metalloproteinase 9 (MMP9) which breaks down the extracellular matrix and basement membranes by preventing its autodegradation and results in tumor progression, invasion and metastasis[15].

Previous in vitro study has shown that upregulation of lipocalin-2 gene may serve as new biomarkers for predicting responsiveness to pemetrexed. In addition, previous studies have reported that expression level of lipocalin-2 may be associated with survival.

In our study, we retrospectively evaluated the lipocalin-2 expression immunohistochemically in sections obtained from paraffin-embedded human lung adenocarcinoma tissue samples and investigated the role of lipocalin-2 expression in predicting treatment response to pemetrexed, and evaluated whether there is a relationship between its expression levels and clinicopathological parameters, including degree of tumor differentiation and driver mutation status in the patients with metastatic lung adenocarcinoma. Survival was also analyzed to determine the prognostic values of lipocalin-2 in these patients.

Methods

Patient and tissue samples

Cases were retrospectively selected from the records of Çanakkale Onsekiz Mart Universtiy Health Research and Practice Hospital and Çanakkale Government Hospital between the years 2015-2019. The study has been approved by the Çanakkale Onsekiz Mart University Ethics Committee. Patients who were diagnosed for adenocarcinoma of the lung and treated second line single agent pemetrexed in the metastatic setting were included into the study. Pemetrexed had been administered every 3 weeks (500 mg/m^2). A total of 30 patients meeting the eligibility criteria were stratified according to treatment response to pemetrexed into two groups as responders and non-responders. The responders group (patients with complete response, partial response and stable disease) and non-responders group (progressive disease) were defined according to the Response Evaluation Criteria in Solid Tumors (RECIST). Moreover, patients were stratified according to the degree of tumor differentiation (as well/moderetely differentiated and poor differentiated) and whether driver mutation was present.

Immunohistochemical evaluation

Formalin-fixed and paraffin-embedded tissue specimens of primary or metastatic lung cancer obtained from pathology department archive of Çanakkale Onsekiz Mart University Health Research and Practice Hospital and Canakkale Government Hospital were used for IHC staining. These specimens were cut (4 μm) and stained with hematoxylin and eosin. A representative slide of each case was stained with lipocalin-2 antibody (Sigma Aldrich clon PA348-26.3.5,1/1000 dilution) in the Leica Bond Max fully automated IHC device (Leica Biosystems). IHC staining was carried out according to the manufacturer's instructions for both the antibody and the device. Stomach tissue was used as positive control (Fig 1d) and a section without primary antibody was used as a negative control. Lipocalin-2 expression was generally cytoplasmic. As bronchus epithelial cells showed weak/moderate staining with lipocalin-2 antibody, lipocalin-2 expression on tumor cells was evaluated according to a score corresponding to the sum of both:(a) staining intensity [0, negative; 1, weak (Fig 1a); 2, intermediate (Fig 1b); and 3, strong (Fig 1c)]; and (b) percentage of positive cells [0, 0% positive cells; 1, <25% positive cells; 2, 26-50% positive cells; and 3, >50% positive

cells], as described elsewhere[16]. The sum of a + b reached a maximum score of 6. Slides were scored in the absence of any clinical data, a score 3 and greater than 3 was evaluated as high expression.

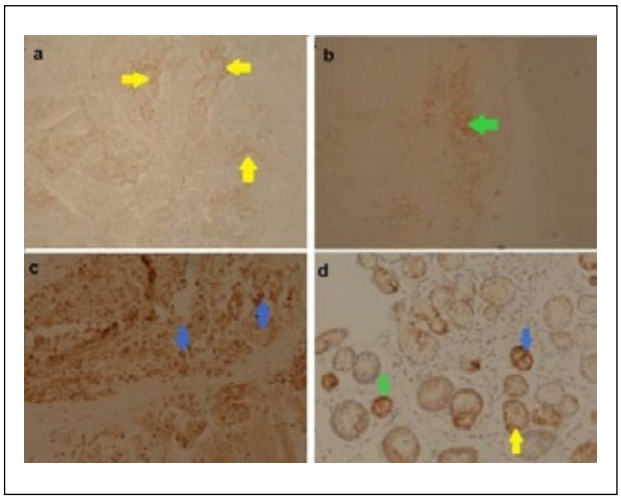

Figure 1: Immunohistochemistry staining using the primary antibody againts lipocalin-2 (**a**) weak (1+, yellow arrow, 100x) (**b**) moderate (2+, green arrow,40x), (**c**) strong (3+, blue arrow, 200x) expression (d) stomach tissue used as positive control (200x).

Statistics

Each clinicopathological variable was compared between the lipocalin-2 positive and negative expression groups, and evaluated with x^2 test or Fisher's exact test. OS time was calculated using the Kaplan-Meier method as the time from the date of diagnosis to the date of death or last follow up. Differences in survival among the groups were compared using the log-rank test. P<0.05 (two-tailed) was considered statistically significant. All statistical analyses were performed using SPSS, version 20.

Results

Lipocalin-2 expression in lung cancer and its relationship to the clinical effectiveness of pemetrexed.

Seventeen tumors (56,6%) showed positive expression for lipocalin-2. Of all patients, 53,3% (16/30) were resistant to pemetrexed. Of lipcalin-2 positive tumors patients 64,7% (11/17) had pemetrexed-responsive disease, compared with 23% (3/13) of lipocalin-2 negative tumors (p=0,028). There was significant association between the lipocalin2- positive and lipocalin2- negative groups with respect to the clinical effectiveness of pemetrexed. The association between the response to pemetrexed and the expressions of lipocalin-2 is summarized in Table 1.

Variable	n	Lipocalin-2 expression		P value
		Low	high	
Responders	14	3	11	0.028
Non-responders	16	10	6	
Well differentiated	17	8	9	0.46
Poor differentiated	13	5	8	
EGFR mutation positive	5	0	5	0,043
EGFR mutation negative	25	13	12	

Table 1: Associations between clinicopathological variables and the expressions of lipocalin-2.

Association of lipocalin-2 expression with tumor differentiation and driver mutation status.

Of lipocalin-2 positive tumors, 47% (8/17) had poor differentiated tumors, compared with 38,5% (5/13) of lipocalin-2 negative tumors (p=0,46) There was no significant association between degree of tumor differentiation and expression of lipocalin-2 protein. Of all patients, 16,6 % (5/30) were positive for EGFR mutation. All five patients with positive EGFR mutation showed high lipocalin-2 expression, compared with 48% (12/25) of patients who do not harbor EGFR mutation (p=0,043) (Table 1).

Association of lipocalin-2 expresssion with survival

PFS was defined as the time from treatment initiation with pemetrexed to disease progression or last visit. All but five patients in study group showed progression at the time of analysis. The longest PFS was 13 months. Median PFS was 7,3 months in patients with lipocalin-2 positive tumors compared to 4,2 months for those with Lipocalin-2 negative tumors (P=0,014).

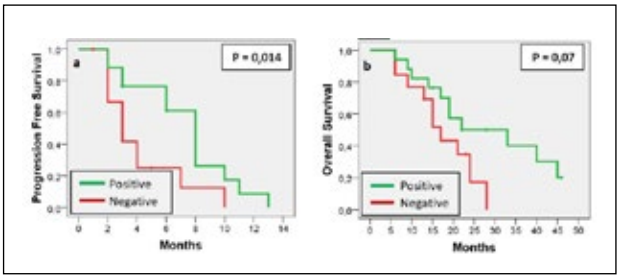

Figure 2: Progression free survival (**a**) and overall survival (**b**) in lipocalin-2 positive and negative patients.

There was significant difference between the two groups with respect to PFS. Twenty-one of 30

patients (70%) died from the date of diagnosis to last follow up. The longest OS was 46 months. The median survival time in patients with positive and negative lipocalin-2 expression was 28,4 and 17,5 months, respectively (log-rank test, p=0,07, Fig. 2).

Discussion

Pemetrexed is a multitarget antifolate metabolite anticancer drug. It affects the synthesis of substrates essential for cell growth and division by inhibiting different enzymes such as thymidylate synthase (TS), glycinamide ribonucleotide formyl transferase (GARFT) and dihydrofolate reductase (DHFR), resulting in cell cycle arrest[17].

Pemetrexed resistance such as other chemotherapeutics is an important problem that reduces the effectiveness of treatment in lung cancer. Little is known about factors for predicting pemetrexed sensitivity or resistance. Resent studies have reported that TS is a biomarker for pemetrexed treatment[18]. When Wu et al examined the level of TS in pemetrexed-sensitive A549 cells and pemetrexed-resistant H1355 cells, they found that it reduced in both cell lines. Therefore, they reported that TS could not be the ideal biomarker for pemetrexed resistance[19].

Moreover, in the above mentioned study, investigator showed that lipocalin-2 expressions increased in response to pemetrexed treatment in a dose-responsive manner in pemetrexed-sensitive A549 cells but not in resistant H1355 cells and reported that lipocalin-2 gene could serve as new biomarkers for predicting responsiveness to pemetrexed[19].

In our clinical study that we performed to comfirm this preclinical observation, we showed that high lipocalin-2 expression resulted in increased pemetrexed sensitivity. There is only one clinical study investigating the predictive role of lipocalin 2 in the current literature. In this study, Wenners et al reported that in low risk subgroups of breast cancer, lipocalin-2 was a predictive marker for pathological complete response (pCR) after neoadjuvant chemotherapy[20].

In previous studies, conflicting results have been reported for the relationship between lipocalin-2 expression and clinicopathologic parmeters including degree of tumor diferantiation, lymph node metastatsis and tumor stage. While ovarian[21], cervical[22], prostate[23] and colorectal cancer[24] studies reported that high level of lipocalin-2 expression was associated with poor differentiated tumors, higher lymph node metastasis and higher tumor stage, conversely, pancreatic canser study[8] showed that positive expression of lipocalin-2 was assosiated with negative lymph node metastatasis and earlier TNM stage. Moreover, in preclinic studies perfomed in colon carcinoma and oral squamous cell carcinoma (OSCC) cell line, have been reported that lipocalin-2 expression correlated inversely with the metastatic potential of these cells[25,26]. In our study, we did not find any association between level of lipocalin-2 expression and tumor differentiation. All patients harboring EGFR mutation showed high lipocalin-2 expression. This result which has not been reported so far in the current literature was particularly interesting. However, it is clear that it should be supported by studies with a larger size of the sample.

Similar divergent results have also been reported for the relationship between lipocalin-2 expression and survival. Pancreatic cancer[8] and OSCC[25] studies showed that positive expression of lipocalin-2 was associated with longer survival time. Conversely, lung, ovarian, cervical, colon and breast cancer studies reported that positive expression of lipocalin-2 was associated with shorter survival time[14,21,22,24,27].

Similar to our study, Ruiz-Morales JM et al also investigated prognostic value of lipocalin-2 in lung adenocarcinoma. They reported that 78 % of tumors showed strong positive staining with lipocalin-2, and that high level of lipocalin-2 expression was associated with shorter survival[14]. In our study 56,6% of lung cancer patient had lipocalin-2 positive tumor. This rate was smaller compared to that reported in the study mentioned above. Moreover, unlike the study of Ruiz-Morales JM et al, we failed to show a significant correlation between expression levels of lipocalin-2 and overall survival although patients with high levels of lipocalin-2 had a marginally longer survival time compared to those with low levels. We aso analyzed PFS for patient taking single agent pemetrexed in metastatic setting and found that high lipocalin-2 expression showed significant corelation with longer PFS time compared to those with negative expression. Our results may reflect some limitation due to presence of censored data, the small size of the sample and interpretation of the IHC evaluation.

In summary, lipocalin-2 was overexpressed in about 56,6% of patients with lung adenocarcinoma. High lipocalin-2 espression was associated with increased pemetrexed sensitivity. There was no significant correlation between lipcalin-2 expression and degree of tumor differantiation, whereas EGFR pos-

itive tumors showed higher lipocalin-2 expression than those with lipocalin-2 negative tumors. Despite no statistical significance, patients with high lipocalin-2 expression tended to have longer overall survival period. In this study, our findings confirmed the preclinical results that high lipocalin-2 expression was associated with increased pemetrexed sensitivity. Despite the relatively limited number of cases, our data imply that lipocalin-2 may be a promising predictive and prognostic marker in patients with lung adenocarcinoma.

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