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Serum 25-hydroxyvitamin D levels correlate with EGFR mutational status in pulmonary adenocarcinoma

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Running head
25(OH)D & EGFR mutation in lung adenocarcinoma
Abstract

There have been several epidemiologic studies about the association between 25(OH)D level and lung cancer risk. We explored the potential association between serum 25-hydroxyvitamin D [25(OH)D] levels and mutations in epidermal growth factor receptor (EGFR) in patients with pulmonary adenocarcinoma. We analyzed clinical data from 135 patients whose serum 25(OH)D levels were measured and EGFR mutational status was tested at the time of diagnosis. The relationship between 25(OH)D and clinical factors such as EGFR mutational status and sex was examined. The median serum 25(OH)D level in patients with pulmonary adenocarcinoma was 16.8 ng/ml (range: 3.0 – 84.3 ng/ml). 25(OH)D level was lower in female patients than male patients ($p = 0.03$). Of interest, 25(OH)D level of patients with EGFR mutated tumors was low compared with those with wild type (median 18.2 ng/ml vs. 14.7 ng/ml, $p = 0.011$). After a dose response relationship between EGFR mutations and 25(OH)D levels (as a continuous variable) was observed (OR = 0.96, $p = 0.036$), we categorized 25(OH)D levels into low ($\leq$ 16.8ng/ml) vs. high (>16.8ng/ml). Multivariate analysis revealed the association between low 25(OH)D levels and high incidence of EGFR mutations (adjusted OR = 2.42, 95% CI: 1.11-5.26, $p = 0.026$). This study suggests that low 25(OH)D levels are associated with EGFR mutations in pulmonary adenocarcinoma.

Keywords: vitamin D, 25-hydroxyvitamin D, EGFR mutation, pulmonary adenocarcinoma

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Introduction

Non-small cell lung cancer (NSCLC) is one of the most fatal cancers worldwide (Jung, et al. 2012; Siegel, et al. 2012) and has been extensively studied, particularly in light of genetic alterations in pulmonary adenocarcinoma. One of the first druggable targets, epidermal growth factor receptor (EGFR) mutations, are predominantly found in pulmonary adenocarcinomas (Shigematsu, et al. 2005). These mutations allow selection of patients who will benefit from these chemotherapeutic agents.

EGFR mutation frequency is inversely related to smoking history (Pham, et al. 2006), which suggests potential associations with etiological factors other than smoking. Ethnic differences in the frequency of EGFR mutations have also been suggested because these mutations are predominantly found in Asians, including Koreans (Bell, et al. 2008). However, despite recent studies that explore contributing factors (Luo, et al. 2012; Tung, et al. 2013), an etiological explanation for EGFR mutations has not yet been proposed.

Vitamin D is a secosteroid that has an important role in bone mineralization by maintaining calcium and phosphorus homeostasis. Vitamin D is biosynthesized from 7-dehydrocholesterol in the skin upon ultraviolet-B (UV-B) exposure. Among the 7-dehydrocholesterol metabolites, 25-hydroxyvitamin D [25(OH)D] is a better indicator of vitamin D status than 1,25(OH)2D because 25(OH)D has a longer half-life and less diurnal variation (Giovannucci 2005; Gray, et al. 1974).

Various factors, including skin pigmentation, physical fitness, diagnostic season, and latitude, determine circulating vitamin D levels (Porojnicu, et al. 2007; Scragg and Camargo 2008). In addition to gender differences, there are geographic variations in vitamin D levels (Mithal, et al. 2009; Scragg and Camargo 2008). The high prevalence of low vitamin D levels in Eastern Asia has been suggested as a cause of osteoporosis in female patients (Lim, et al. 2008). A previous Korean study also reported a high incidence of vitamin D deficiency, which was more prominent in females (Choi, et al.).

Experimental studies suggest that vitamin D has anti-proliferation and pro-differentiation effects in solid tumors (Fleet 2008). Recent in vitro data have shown EGFR mutant adenocarcinoma cell lines may be
sensitive to 1,25(OH)₂D, the final activating form of vitamin D (Zhang, et al.). Furthermore, an epidemiological study reported that low vitamin D intake is associated with higher risks of lung cancer in women who have never smoked, an EGFR mutation-rich population (Cheng, et al.). Investigators have also revealed that high circulating vitamin D levels are associated with lower risks of lung cancer in females (Kilkkinen, et al. 2008), which reflects its link to a specific subtype of lung cancer.

The demographic features of the vitamin D deficient population (Choi et al.; Scragg, et al. 1995) suggests that patients with vitamin D deficiencies are likely to harbor EGFR-mutant tumors. However, to our knowledge, there are few studies that indicate a different prevalence of EGFR mutations according to circulating vitamin D levels. Thus, we explored the potential association of low serum 25(OH)D levels with the frequency of EGFR mutations in patients with pulmonary adenocarcinomas.

**Patients and methods**

**Patients**

A total of 135 patients who were diagnosed with lung adenocarcinoma and whose serum 25(OH)D levels were evaluated were consecutively enrolled between January 2011 and August 2013 at the Korea Cancer Center Hospital. Patients who had results for EGFR mutation were identified from NSCLC pathology database of our institution and included in our study. Tumor stage was classified using the recently revised TNM system proposed by American Joint Committee on Cancer (7th edition). Serum 25(OH)D levels were measured at the time of diagnosis. Patients with squamous cell and adenosquamous carcinoma were excluded.

**Determination of 25(OH)D Levels**

Serum samples obtained from patients were maintained at 2-8°C and assayed daily in workdays not to exceed 48 hours since the sampling of patients’ serum. Modified radioimmunoassays (RIA) to incorporate ¹²⁵I-labeled reporters and calibrators into a serum matrix were used to quantitatively measure 25(OH)D levels in serum (DIAsource ImmunoAssays S.A., Louvain-la-Neuve, Belgium). Statistics of this RIA assay suggest acceptable reproducibility compared to previous methods (Zerwekh 2008).

The RIA was performed in separate laboratory using the method of manufacturer’s recommendations. A
fixed amount of $^{125}$I-labeled 25(OH)D competes with 25(OH)D from either extracted serum samples, controls, or calibrators for a fixed number of specific antibody sites immobilized to the lower and inner surface of plastic tubes. After incubations for 2 hours, tubes are washed and counted using a gamma counter (Hollis, et al. 1993). The amount of gamma radiation measured is inversely proportional to the 25(OH)D concentration. A six point non-linear calibration curve was used to determine 25(OH)D.

**EGFR Genotyping**

Genomic DNA was extracted from paraffin-embedded tumor tissues. In patients whose only available tissue was the cytological sample at initial diagnosis, methanol-fixed cytological specimens were used for DNA extraction (Boldrini, et al. 2007). Pyrosequencing was performed to detect EGFR mutations, as described previously (Na, et al. 2011).

**Statistical Methods**

Statistical analyses were performed using STATA version 11 (Stata Corp, College Station, TX, USA). To compare serum 25(OH)D levels as continuous variables according to different subgroups, Mann-Whitney U tests were used. A median value was used as the cut-off criterion for converting to categorical variables in sequential analyses using chi-square and logistic regression tests. Associations between 25(OH)D levels and clinical features (age, sex, smoking, TNM stage, and metastatic site) as well as EGFR mutational status were analyzed using logistic regression analyses. Clinical factors found to be associated with 25(OH)D levels in univariate analyses were included in the multivariate analyses. $p$ values <0.05 (two-sided) were considered statistically significant.

**Ethics**

The Institutional Review Board of the Korea Cancer Center Hospital reviewed and approved this study protocol. The recommendations of the Declaration of Helsinki for biomedical research involving human subjects were followed.

**Results**

**Patient Characteristics**
A total of 135 patients (71 men and 64 women) with a median age of 64 years (range, 33–86 years) were consecutively enrolled in our study. All patients had adenocarcinoma as the histologic subtype. Approximately half of the patients (58 patients, 43.0%) had metastatic disease, whereas 77 patients (57.0%) had non-metastatic disease. Sixty-seven patients (49.6%) had a history of smoking (86.6% in male patients and 13.4% in female patients, respectively), and 59 patients (43.7%) harbored EGFR mutations (Table 1). Deletion in exon 19 (28/59 patients, 47.5%) and L858R mutation in exon 21 (27/59 patients, 45.8%) were the most commonly observed mutations. The remaining 4 patients (6.8%) had a G719X mutation in exon 18.

**Serum 25(OH)D Levels**

The median serum 25(OH)D level in patients with pulmonary adenocarcinoma was 16.8 ng/ml (range: 3.0 – 84.3 ng/ml). The mean 25(OH)D levels were slightly lower in patients who were initially diagnosed during the winter season; however, it was not statistically significant (median: 15.6 ng/ml in winter vs. 17.1 ng/ml in non-winter, *p* = 0.44).

Females had lower serum 25(OH)D levels than males (median: 15.4 ng/ml in female patients vs. 17.9 ng/ml in male patients, *p* = 0.027). In addition, 25(OH)D levels did not differ according to stage (median: 16.6 ng/ml in patients with early stage disease vs. 17.6 ng/ml in patients with advanced disease, *p* = 0.73).

**25(OH)D Levels and EGFR Mutation**

In our study cohort, female patients had higher frequencies of EGFR mutation compared to male patients [frequency of EGFR mutation: 64.1% (41/64) in females vs. 25.4% (18/71) in males, *p* < 0.001]. Smoking was also inversely correlated to EGFR mutation [63.2% (43/68) in non-smokers vs. 23.9% (16/67) in smokers, *p* <0.001].

Serum 25(OH)D levels in patients with pulmonary adenocarcinoma harboring EGFR mutations were significantly lower compared to patients with wild type EGFR (median 14.7 ng/ml, 95% CI: 14.4 – 19.4 vs. median 18.2 ng/ml, 95% CI: 18.4 – 24.1, *p* = 0.011) (Figure 1). After a dose response relationship between EGFR mutations and serum 25(OH)D levels (as a continuous variable) was observed (OR = 0.96,
95% CI: 0.93 – 1.00, \( p = 0.036 \), we categorized vitamin D as a binary variable using the median value as a cut-off point (16.8 ng/ml).

When the 25(OH)D level was divided into low (\( \leq 16.8\)ng/ml) and high (>16.8ng/ml), the prevalence of EGFR mutations was more common in patients with low 25(OH)D levels compared to those with high 25(OH)D levels (57.4% vs. 29.9%, \( p = 0.001 \)). Importantly, the inverse association between serum 25(OH)D levels and EGFR mutation remained significant in the multivariate analysis (adjusted OR = 2.42, 95% CI: 1.11-5.26, \( p = 0.026 \)) (Table 2).

**Discussion**

To our knowledge, this is the first study to suggest an inverse association between serum 25(OH)D levels and the prevalence of EGFR mutations. We observed EGFR mutations were more frequent in patients with low vitamin D levels at their initial diagnosis. The predominance of EGFR mutations in patients with low vitamin D levels was significant considering gender and smoking, which are well known predictors of mutational status (Shigematsu et al. 2005).

Recent studies have suggested a protective effect of vitamin D in several solid tumors, including colon and breast cancer (Mohr, et al.; Pereira, et al.). In a mouse model, a low incidence of pulmonary tumors with 1,25(OH) vitamin D intake has also been demonstrated (Mernitz, et al. 2007). Interestingly, investigators have reported protective effects of vitamin D for lung cancer risk in older women with no history of smoking (Cheng et al.), a subpopulation with a high likelihood of EGFR-mutated tumors (Cho, et al. 2012; Zhang, et al.). Notably, based on epidemiological data, an inverse association of vitamin D levels with lung cancer risk has been observed in females, but not in males (Kilkkinen et al. 2008; Weinstein, et al.). Thus, this finding supports our hypothesis that low vitamin D levels might lead to an increased incidence of EGFR-mutant lung cancer.

Several Asian investigators have suggested the contribution of infection, including human papillomavirus (HPV) and tuberculosis, to EGFR mutant tumors (Luo et al. 2012; Tung et al. 2013). In a previous study that included squamous cell carcinomas, EGFR mutations were frequently observed in HPV-positive...
tumors (41% vs. 20% in HPV-negative tumors, \( p = 0.006 \)); however, adjusted significance did not remain \( (p = 0.111) \) (Tung et al. 2013). In another study including some non-classical EGFR mutant tumors, old tuberculosis lesions were more frequently observed in EGFR-mutant tumors compared to wild type tumors (80.6% vs. 65.5%, \( p = 0.018 \)). In contrast to these studies, we included a relatively homogenous population of pulmonary adenocarcinomas and analyzed data based on classic EGFR mutations. Notably, a large portion of the EGFR-mutant tumors (approximately 57% in our data) was detected in patients with low vitamin D levels. Furthermore, we observed a significant association between EGFR mutations and vitamin D levels in the adjusted model \( (p = 0.026) \). Interestingly, an immunomodulatory effect of vitamin D on infection has been suggested through antimicrobial peptide production for respiratory viruses, or tuberculosis (Liu, et al. 2006; Phelan 1947). In theory, vitamin D may play a role in the potential contribution of infection to EGFR mutations. Further comprehensive studies are required to test this hypothesis.

Although we observed an inverse association between EGFR mutation incidence and vitamin D levels, the causative relationship is uncertain. However, the protective function of vitamin D against genetic damage has been suggested (Nair-Shalliker, et al. 2012). In other experimental studies of lung cancer (Hershberger, et al. 1999; Nakagawa, et al. 2005), anti-carcinogenic activities of vitamin D, including inhibiting cell proliferation and promoting apoptosis, have been demonstrated. Clinical data have also revealed an association between low vitamin D levels and shortened telomere lengths, a potential risk factor for lung cancer (Richards, et al. 2007; Willeit, et al. 2010). Furthermore, molecular evidence of enhanced catabolic inactivation and growth inhibition to vitamin D in EGFR-mutant cells supports an inhibitory role of vitamin D, particularly in EGFR-mutant tumors (Zhang, et al. 2013). Taken together, these data suggest it is likely that low vitamin D levels may initiate a favorable milieu for the development of EGFR-mutant tumors.

Vitamin D might not be considered in the direct relationship of disease, but rather as a marker of physical activity, which is directly linked to sun exposure (Scruggs and Camargo 2008). In particular, cautious interpretation is required regarding the prognostic impact of vitamin D (Dizdar, et al. 2008; Giovannucci, et al. 2006; Mei, et al. 2007). Similarly, based on previous studies suggesting that females have low
vitamin D levels compared to males (Arabi, et al. 2010; Choi, et al. 2011), the gender difference might explain the inverse association of vitamin D level and EGFR mutational status. However, it should be stressed that the significance of vitamin D levels regarding EGFR mutations has maintained after adjustment of other factors including gender in the present study (Table 2). Therefore, it is less likely that the significance could be biased by gender difference.

A recent study of vitamin D and related binding proteins found that its bioavailability should be considered in defining deficiency in different ethnicities (Powe, et al. 2013). However, this does not affect our study because our population was of the same ethnicity. Compared to the representative values from nationwide data (greater than 20.6 ng/ml and 17.3 ng/ml for males and females, respectively, older than 30 years), a significant number of patients in this study (median 17.9 ng/ml and 15.4 ng/ml for males and females, respectively) are likely to be considered vitamin D deficient (Choi et al. 2011). Considering the potential prognostic significance of low vitamin D levels in early stage lung cancer (Mei et al. 2007), vitamin D supplements can be issued in this population. However, the beneficial effects of vitamin D supplements, as well as appropriate dosages, have not yet been determined.

The causative factors of lung cancers have not been fully understood, particularly in never-smokers.. Despite the association between low level of 25(OH)D and EGFR mutation, it is unknown whether it is causative or not. This result might be biased by unexpected factors such as radon exposure in residences, which is expected to be high in patients with indoor life style. However, according to one retrospective Western study (Taga, et al. 2012), genetic difference by radon exposure has not been suggested. Considering complexity in carcinogenic process in lung cancer, further molecular studies in diverse ethnicity need to be followed.

Despite the limitations of a small-sized, retrospective study, our study is the first to report an association between serum 25(OH)D levels and EGFR mutations. This study also suggests that further molecular data are needed in epidemiological studies to prove the correlation between vitamin D levels and the risk of lung cancer. Larger prospective studies with evaluation of 25(OH)D related proteins and genetic diversity should be followed.
Acknowledgement

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Conflict of Interest Statement

The authors have declared no conflicts of interest.
References


Dizdar O, Bulut N & Altundag K 2008 Vitamin D intake may be a predictor of response to aromatase inhibitors in postmenopausal women with hormone receptor positive breast cancer. *Breast Cancer Res Treat* **109** 403.


Giovannucci E, Liu Y & Willett WC 2006 Cancer incidence and mortality and vitamin D in bla


vitaminosis D. *Osteoporos Int* **20** 1807-1820.


Scruggs R & Camargo CA, Jr. 2008 Frequency of leisure-time physical activity and serum 25-hyroxvitamin D levels in the US population: results from the Third National Health and Nutrition


Figure legends

**Figure 1.** Circulating 25(OH)D levels according to EGFR mutational status in patients with pulmonary adenocarcinoma (median 18.2 ng/ml, 95% CI: 18.4 – 24.1 vs. median 14.7 ng/ml, 95% CI: 14.4 – 19.4, $p = 0.011$)
Table 1. Baseline characteristics (n = 135)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number of patients (%)</th>
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<tbody>
<tr>
<td>Age, years</td>
<td>Median 64</td>
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<tr>
<td></td>
<td>Range 33-86</td>
</tr>
<tr>
<td>Sex</td>
<td>Male 71 (52.6)</td>
</tr>
<tr>
<td></td>
<td>Female 64 (47.4)</td>
</tr>
<tr>
<td>Smoking</td>
<td>Yes 67 (49.6)</td>
</tr>
<tr>
<td></td>
<td>No 68 (50.4)</td>
</tr>
<tr>
<td>Stage</td>
<td>Non-metastatic 77 (57.0)</td>
</tr>
<tr>
<td></td>
<td>Metastatic 58 (43.0)</td>
</tr>
<tr>
<td>EGFR Status</td>
<td>Wild type 76 (56.3)</td>
</tr>
<tr>
<td></td>
<td>EGFR mutation 59 (43.7)</td>
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Table 2. EGFR mutation and its association with clinical factors including 25(OH)D level

<table>
<thead>
<tr>
<th></th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>p value</td>
</tr>
<tr>
<td>Sex (female)</td>
<td>5.25 (2.51-11.00)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.18 (0.08-0.39)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stage (metastasis)</td>
<td>0.75 (0.38-1.49)</td>
<td>0.41</td>
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<tr>
<td>25(OH)D (≤16.8ng/ml)</td>
<td>3.16 (1.55-6.43)</td>
<td>0.002</td>
</tr>
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</table>
Circulating 25(OH)D levels according to EGFR mutational status in patients with pulmonary adenocarcinoma (median 18.2 ng/ml, 95% CI: 18.4 – 24.1 vs. median 14.7 ng/ml, 95% CI: 14.4 – 19.4, p = 0.011)