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

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Abstract
There have been several epidemiological studies of the association between 25-hydroxyvitamin D (25(OH)D) level and lung cancer risk. We explored the potential association between serum 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). The level of 25(OH)D was lower in female patients than in male patients (P<0.03). Interestingly, 25(OH)D levels of patients with EGFR-mutated tumors were low compared with those with wild-type tumors (median 18.2 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 as low (≤16.8 ng/ml) and high (>16.8 ng/ml). Multivariate analysis revealed the association between low 25(OH)D levels and a high incidence of EGFR mutations (adjusted OR=2.42, 95% CI: 1.11–5.26, P=0.026). The results from this study indicate that low 25(OH)D levels are associated with EGFR mutations in pulmonary adenocarcinoma.

Key Words
- vitamin D
- 25-hydroxyvitamin D
- EGFR mutation
- pulmonary adenocarcinoma

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 the light of genetic alterations in pulmonary adenocarcinoma. One of the first druggable targets, epidermal growth factor receptor (EGFR) mutations, is 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 indicates 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 have explored 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 u.v.-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 (Gray et al. 1974, Giovannucci 2005).

Various factors, including skin pigmentation, physical fitness, diagnostic season, and latitude, determine circulating vitamin D levels (Porojnicu et al. 2007, Scragg & Camargo 2008). In addition to sex differences, there are geographical variations in vitamin D levels (Scragg & Camargo 2008, Mithal et al. 2009). The high prevalence of low vitamin D levels in Eastern Asia has been suggested to be a cause of osteoporosis in female patients (Lim et al. 2008). A previous Korean study has also reported a high incidence of vitamin D deficiency, which was more prominent in females (Choi et al. 2011).

Results from experimental studies indicate that vitamin D has antiproliferation and pro-differentiation effects in solid tumors (Fleet 2008). Recent in vitro data have indicated that EGFR-mutant adenocarcinoma cell lines may be sensitive to 1,25(OH)2D, the final activating form of vitamin D (Zhang et al. 2013). Furthermore, an epidemiological study demonstrated 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. 2013). 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 (Scragg et al. 1995, Choi et al. 2011) indicate 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 in relation 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 mutations were identified from NSCLC pathology database of our institution and included in our study. Tumor stage was classified using the recently revised tumor-node-metastasis (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 on workdays less than 48 h after the sampling of patients’ serum. Modified RIAs 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 for this RIA indicate acceptable reproducibility compared with methods used previously (Zerwekh 2008).

The RIA was performed in a separate laboratory according to the manufacturer’s recommendations. A fixed amount of 125I-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 surfaces of plastic tubes. After incubation for 2 h, tubes were washed and counted using a γ counter (Hollis et al. 1993). The amount of γ 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, the Mann–Whitney U test was used. A median value was used as the cut-off criterion for conversion to categorical variables in sequential analyses using the $\chi^2$ 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 histological subtype. Approximately half of the patients (58 patients, 43.0%) had metastatic disease, whereas 77 patients (57.0%) had non-metastatic disease. Of the total number of patients, 67 (49.6%) had a history of smoking (86.6% of male patients and 13.4% of female patients respectively), and 59 (43.7%) harbored EGFR mutations (Table 1). Deletions in exon 19 (28/59 patients, 47.5%) and the L858R mutation in exon 21 (27/59 patients, 45.8%) were the most commonly observed mutations. The remaining four 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 winter; however, the difference was not statistically significant (median: 15.6 ng/ml in winter versus 17.1 ng/ml during other seasons, $P=0.44$).

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

Serum 25(OH)D levels and EGFR mutation

In our study cohort, female patients had higher frequencies of EGFR mutations compared with male patients (frequency of EGFR mutation: 64.1% (41/64) in females versus 25.4% (18/71) in males, $P<0.001$). Smoking was also inversely correlated with EGFR mutation (63.2% (43/68) in non-smokers versus 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 with 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, $P=0.011$; Fig. 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 classified as low ($\leq16.8$ ng/ml) or high ($>16.8$ ng/ml), the prevalence of EGFR mutations was more common in patients with low 25(OH)D levels compared with those with high 25(OH)D levels (57.4 versus 29.9%, $P=0.001$). Importantly, the inverse association between serum 25(OH)D levels and

<table>
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<th>Table 1 Baseline characteristics ($n=135$)</th>
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<td><strong>Age (years)</strong></td>
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<td>Range</td>
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<td><strong>Sex</strong></td>
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<tr>
<td>Male</td>
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<tr>
<td>Female</td>
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<tr>
<td><strong>Smoking</strong></td>
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<tr>
<td>Yes</td>
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<tr>
<td>No</td>
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<tr>
<td><strong>Stage</strong></td>
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<td>Non-metastatic</td>
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<td>Metastatic</td>
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<td><strong>EGFR status</strong></td>
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<td>Wild-type</td>
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<td>EGFR mutation</td>
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EGFR mutation remained significant in the multivariate analysis (adjusted OR $Z 2.42$, 95% CI: 1.11–5.26, $P$ $Z 0.026$; Table 2).

Discussion

To our knowledge, this is the first study to indicate an inverse association between serum 25(OH)D levels and the prevalence of EGFR mutations. We observed that 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 sex and smoking, which are well-known predictors of mutational status (Shigematsu et al. 2005).

Results from recent studies have indicated a protective effect of vitamin D in several solid tumors, including colon and breast cancers (Mohr et al. 2012, Pereira et al. 2012). 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 the protective effects of vitamin D against lung cancer risk in older women with no history of smoking (Cheng et al. 2013), a subpopulation with a high likelihood of EGFR-mutated tumors (Cho et al. 2012, Zhang et al. 2012). 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. 2011). 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 versus 20% in HPV-negative tumors, $P=0.006$); however, the adjusted results did not remain significant ($P=0.111$; 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 with wild-type tumors (80.6 versus 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 proportion of the EGFR-mutant tumors (approximately 57% in the present study) were 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

Table 2  EGFR mutation and its association with clinical factors including 25(OH)D level

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<th>Univariate</th>
<th>Multivariate</th>
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<tr>
<td></td>
<td>OR (95% CI)</td>
<td>$P$ value</td>
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<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 (0.086–0.39)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Stage (metastasis)</td>
<td>0.75 (0.38–1.49)</td>
<td>0.41</td>
</tr>
<tr>
<td>25(OH)D ($\leq 16.8$ ng/ml)</td>
<td>3.16 (1.55–6.43)</td>
<td>0.002</td>
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on infection has been suggested through production of antimalarial peptide against respiratory viruses or tuberculosis (Phelan 1947, Liu et al. 2006). 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, a 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), antitumorigenic 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 in response 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 results indicate 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 to be directly related to disease, but rather as a marker of physical activity, which is directly linked to sun exposure (Scragg & Camargo 2008). In particular, cautious interpretation is required regarding the prognostic value of vitamin D (Giovannucci et al. 2006, Mei et al. 2007, Dizdar et al. 2008). Similarly, on the basis of results from previous studies indicating that females have low vitamin D levels compared with males (Arabi et al. 2010, Choi et al. 2011), the sex difference might explain the inverse association of vitamin D level and EGFR mutational status. However, it should be emphasized that the significance of vitamin D levels with respect to EGFR mutations was maintained after adjustment for other factors including sex in this study (Table 2). Therefore, it is less likely that the significance could be biased by sex differences.

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 with the representative values from nationwide data (>20.6 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 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 are not fully understood, particularly in never-smokers. Despite the association between low levels of 25(OH)D and EGFR mutation, it is not known 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 an indoor lifestyle. However, according to one retrospective Western study (Taga et al. 2012), genetic difference due to radon exposure were not indicated. Considering the complexity of the carcinogenic process in lung cancer, there is a need for further molecular studies in patients with diverse ethnicity.

Despite the limitations of a small-sized, retrospective study, our study is the first, to our knowledge, to report an association between serum 25(OH)D levels and EGFR mutations. The results of this study also indicate 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 carried out.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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