Somatic Mutations of ERBB2 Kinase Domain in Gastric, Colorectal, and Breast Carcinomas

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Abstract

Purpose: Recent reports revealed that the kinase domain of the ERBB2 gene is somatically mutated in lung adenocarcinoma, suggesting the mutated ERBB2 gene as an oncogene in human cancers. However, because previous reports focused the mutational search of ERBB2 primarily on lung cancers, the data on ERBB2 mutations in other types of human cancers have been largely unknown.

Experimental Design: Here, we did a mutational analysis of the ERBB2 kinase domain by PCR single-strand conformational polymorphism assay in gastric, colorectal, and breast carcinoma tissues.

Results: We detected the ERBB2 kinase domain mutations in 9 of 180 gastric carcinomas (5.0%), in 3 of 104 colorectal carcinomas (2.9%), and in 4 of 94 breast carcinomas (4.3%). All of the detected ERBB2 mutations except for one in-frame deletion mutation were missense mutations. Of the 16 ERBB2 mutations detected, 4 affected Val1777 in the exon 20 site, and 3 affected Leu785 in the exon 19 site. We simultaneously analyzed the somatic mutations of EGFR, K-RAS, PIK3CA, and BRAF genes in the 16 samples with ERBB2 mutations, and found that all of the 3 colorectal carcinoma samples with ERBB2 mutations harbored K-RAS mutations.

Conclusion: This study showed that in addition to lung adenocarcinomas, ERBB2 kinase domain mutation occurs in other common human cancers such as gastric, breast, and colorectal cancers, and suggested that alterations of ERBB2-mediated signaling pathway by ERBB2 mutations alone or together with K-RAS mutations may contribute to the development of human cancers.

Protein tyrosine kinases, which regulate cell signaling pathways mediating a number of processes in cell survival and growth, comprise a large fraction of the dominant oncogenes known to date (1). The epidermal growth factor receptor (EGFR) is a prototypical receptor protein tyrosine kinase, and the EGFR gene is the mammalian equivalent of avian viral oncogene v-erb (2). Because of their importance in tumorigenesis, receptor tyrosine kinases are popular rational targets for cancer therapy (1). Recent studies showed that lung adenocarcinomas possessed EGFR gene mutations (3–6). Most importantly, the ERBB2 gene mutations in lung cancers could predict significant clinical responses to gefitinib (Iressa) and erlotinib (Tarceva), orally active EGFR tyrosine kinase inhibitors, which have given significant clinical benefit to a subset of lung cancer patients (3–5). The EGFR mutations have been detected only in the exons that encode the intracellular kinase domain (3–6).

The EGFR family consisted of four receptor tyrosine kinases, EGFR (ERBB1/HER1), ERBB2 (HER2), ERBB3 (HER3), and ERBB4 (HER4; refs. 1, 2, 7, 8). What they possess in common are their extracellular ligand-binding domain, a transmembrane domain, a tyrosine kinase domain, and a regulatory domain. Binding of the EGFR family members with their ligands enables the formation of homodimers or heterodimers with other EGFR family members, which subsequently results in the phosphorylation of intracellular tyrosine residues, activating diverse signaling pathways. The high sequence homologies and the presence of heterodimerization among the EGFR family members indicate that the EGFR signaling pathway is not linear, and suggests the possibility that other EGFR members besides EGFR might possess activating mutations in human cancers.

ERBB2 overexpression by the ERBB2 gene amplification is present in ~25% of invasive breast cancers, and is associated with poor prognosis of the patients (9, 10). The humanized antibody trastuzumab (Herceptin) against the overexpressed ERBB2 is proven to be effective in treating breast cancers with ERBB2 amplification (9, 10). Recently, Stephens et al. (11) reported that 10% of the lung adenocarcinomas harbored ERBB2 somatic mutations in the DNA sequences encoding the...
kinase domain. Another report also confirmed that the ERBB2 kinase domain was somatically mutated in lung adenocarcinomas (12). Stephens et al. also identified ERBB2 mutations in one gastric (1 of 20), one ovarian (1 of 27), and one brain (1 of 10) tumor. However, because previous studies focused the ERBB2 mutation screening mainly on lung cancers and analyzed small numbers of samples in other types of cancers (11, 12), further studies to detect ERBB2 mutations in other types of cancers are now needed to understand the role of ERBB2 mutations in the development of human cancers. In the present study, we analyzed somatic mutations of the ERBB2 kinase domain in the tissue samples from gastric, breast, and colorectal carcinomas by PCR-based single-strand conformational polymorphism (SSCP) analysis.

Materials and Methods

Tissue samples. Methacarn-fixed tissues of 180 gastric, 94 breast, and 104 colorectal carcinomas were randomly selected for the study. All of the patients were Asians (Korean). Approval was obtained from the Catholic University of Korea, College of Medicine’s Institutional Review Board for this study. Informed consent was provided according to the Declaration of Helsinki. The gastric carcinomas consisted of 79 diffuse-type, 63 intestinal-type, and 38 mixed-type gastric adenocarcinomas according to Lauren’s classification, and 40 early and 140 advanced gastric carcinomas according to the depth of invasion. The tumor-node-metastasis (TNM) stages of the gastric carcinomas were classified as stage 0 (19), stage I (23), stage II (50), stage III (40), and stage IV (15). The breast carcinomas consisted of 15 intraductal and 79 invasive ductal carcinomas. The TNM stages of the breast carcinomas were classified as stage 0 (15), stage I (8), stage II (50), and stage IV (21). The colorectal carcinomas originated from cecum (n = 2), ascending colon (n = 19), descending colon (n = 4), transverse colon (n = 6), sigmoid colon (n = 28), and rectum (n = 45). The TNM stages of the colorectal carcinomas were classified as stage I (10), stage II (48), stage III (36), and stage IV (10). We analyzed the primary tumors, but not the metastatic lesions. We did not include the cancer cell lines in this study. Tumor cells and normal cells from the same patients were selectively procured from H&E-stained slides using a 30-gauge 1/2 hypodermic needle (Becton Dickinson, Franklin Lakes, NJ) affixed to a micromanipulator by microdissection, as described previously (13). DNA extraction was done by a modified single-step DNA extraction method by proteinase K treatment, as described previously (13).

SSCP analysis and DNA sequencing. To date, all of the ERBB2 mutations in cancers were detected within the DNA sequences encoding the kinase domain (11, 12). Thus, we analyzed the ERBB2 mutations in exons 18 to 23 encoding the kinase domain. Genomic DNA from each of the tumor cells and normal cells were amplified with seven primer pairs covering exons 18 to 23. The primer sequences were as follows (forward and reverse, respectively): exon 18 (5'-gacacagtggagggtcgtc-3' and 5'-atctaaagcttgccacacca-3'), exon 19 (5'-gccagcctttctca-a-3' and 5'-atccggcttctccgc-3'), exon 20 (5'-gtctgttggaggtgcggtcgtc-3' and 5'-ctctgcaccctggaggtcgtc-3'), exon 21 (5'-gctgttcctgacggtcggtcgtc-3' and 5'-ctctgcaccctggaggtcgtc-3'), exon 22 (5'-ggccacccagcagcctggaggtcgtc-3' and 5'-ctctgcaccctggaggtcgtc-3'), and exon 23 (5'-ctctgcaccctggaggtcgtc-3' and 5'-ctctgcaccctggaggtcgtc-3'). Numbering of cDNA of ERBB2 was done with respect to the ATG start codon (NM_004448). Radioisotope (32PdCTP) was incorporated into the PCR products for detection by autoradiogram. The PCR reaction mixture was denatured for 1 minute at 94°C and incubated for 30 cycles (denaturing for 30 seconds at 94°C, annealing for 30 seconds at 50°C to 60°C, and extending for 30 seconds at 72°C). Other procedures such as PCR and SSCP analysis were done as described previously (14, 15). After SSCP, DNAs showing mobility shifts were cut out from the dried gel, and reamplified for 30 cycles using the same primer sets. Sequencing of the PCR products was carried out using the cyclic sequencing kit (Perkin-Elmer, Foster City, CA) according to the manufacturer’s recommendation.

EGFR (exons 18-21), K-RAS (codons 12 and 13), PIK3CA (exons 9 and 20), and BRAF (exons 11 and 15) mutation data from the present study, breast, and colorectal carcinomas by PCR-based single-strand conformational polymorphism (SSCP) analysis.
For ERBB2 hybridization, LSI HER2/CEP17 probes (Vysis, Inc., Downers Grove, IL) was applied, and a coverslip was placed. After overnight hybridization at 37 °C in a humidified chamber, the slides were washed with 72 °C posthybridization wash buffer for 2 minutes. Nuclei were counterstained with 4,6-diamino-2-phenylindole. The fluorescence in situ hybridization procedures were done as described previously (19).

**Results**

Genomic DNAs isolated through microdissection were analyzed for the detection of mutations in the exons and the exon-intron junctions of ERBB2 gene encoding the kinase domain by the PCR-SSCP analysis. Enrichment and DNA sequencing analysis of aberrantly migrating bands on the SSCP led to the identification of 16 ERBB2 kinase domain mutations out of the 378 samples (4.2%; Fig. 1; Table 1). None of the normal samples from the same patients showed evidence of mutations by the SSCP (Fig. 1), indicating that the mutations originated somatically. The 16 mutations consisted of 15 missense and 1 deletion mutation, and were detected in exon 18 (2 mutations), exon 19 (6 mutations), exon 20 (5 mutations), exon 21 (2 mutations), and exon 22 (1 mutation; Fig. 2). The mutations were identified in 9 of the 180 gastric carcinomas (5.0%), in 4 of the 94 breast carcinomas (4.3%), and in 3 of the 104 colorectal carcinomas (2.9%). According to the SSCP (Fig. 1), whereas most cancers with the mutations (15 of 16) showed wild-type bands and additional aberrant bands, one breast carcinoma showed only two aberrant bands without wild-type bands, indicating either homozygous mutation or hemizygous mutation with allelic loss (Fig. 1A, breast case 43). There was no significant correlation of the ERBB2 mutation with the degree of differentiation or the TNM stage of the tumors or the gender of the patients (Fisher's exact test, P > 0.05; Table 1). Also, there was no significant correlation of the ERBB2 mutation with the histologic subtype of the gastric carcinomas (Lauren's classification) and the anatomic location of the colorectal carcinomas (Fisher's exact test, P > 0.05; Table 1).

The ERBB2 mutations were detected in advanced gastric carcinomas (9 of 140), but not in the early gastric carcinomas (0 of 40). Similarly, in breast carcinomas, only the invasive ductal carcinomas, but not the intraductal carcinomas (0 of 15) harbored the ERBB2 mutations (4 of 79). However, the relationship between the incidence of ERBB2 mutation and the progression of these tumors (early gastric carcinomas versus advanced gastric carcinomas, intraductal versus invasive breast carcinomas) was not statistically significant (Fisher's exact test, P > 0.05). We repeated the experiments thrice, including PCR, SSCP, and DNA sequencing analysis to ensure the specificity of the results, and found that the data were consistent (data not shown).

### Table 1. Summary of the ERBB2 mutations of the gastric, breast and colorectal cancers

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age/sex</th>
<th>TNM stage</th>
<th>Primary tumor (site)</th>
<th>Associated mutations</th>
<th>Mutation type</th>
<th>Mutation site</th>
<th>Nucleotide change</th>
<th>Predicted amino acid changes</th>
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<tbody>
<tr>
<td>Stomach 205</td>
<td>M/63</td>
<td>II</td>
<td>AGC</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Stomach 262</td>
<td>M/70</td>
<td>II</td>
<td>AGC</td>
<td>--</td>
<td>--</td>
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<td>IV</td>
<td>AGC</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Stomach 213</td>
<td>M/70</td>
<td>IB</td>
<td>AGC</td>
<td>--</td>
<td>--</td>
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</tr>
<tr>
<td>Stomach 97</td>
<td>F/55</td>
<td>IB</td>
<td>AGC</td>
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<td>--</td>
<td>--</td>
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<tr>
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<td>M/71</td>
<td>IIIA</td>
<td>AGC</td>
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<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
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<td>II</td>
<td>AGC</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Stomach 287</td>
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<td>IB</td>
<td>AGC</td>
<td>--</td>
<td>--</td>
<td>--</td>
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<td>--</td>
</tr>
<tr>
<td>Colon 43</td>
<td>F/73</td>
<td>IIA</td>
<td>ADENO (rectum)</td>
<td>G12D</td>
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<td>--</td>
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<tr>
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<td>F/74</td>
<td>IIA</td>
<td>ADENO (rectum)</td>
<td>G12D</td>
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<tr>
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<tr>
<td>Breast 51</td>
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<tr>
<td>Breast 151</td>
<td>F/55</td>
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<td>F/45</td>
<td>I</td>
<td>invasive ductal</td>
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<td>--</td>
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</tbody>
</table>

**Abbreviations:** AGC, advanced gastric cancer; ADENO, adenocarcinoma.
We also analyzed K-RAS, BRAF, PIK3CA, and EGFR gene mutations in the same tissue samples. We detected PIK3CA mutations in the gastric, colorectal, and breast carcinomas, and BRAF mutations in the gastric and colorectal carcinomas (17, 18), but the tumors with either BRAF or PIK3CA mutations did not harbor any ERBB2 kinase domain mutation. We could not find any EGFR mutation in the gastric, colorectal, and breast cancers (16). K-RAS mutations were observed in the colorectal (32 of 104 samples) and gastric carcinomas (6 of 180 samples). In gastric cancers, the nine tumors with ERBB2 mutation did not harbor any K-RAS mutation. By contrast, in the colorectal cancers, all of the three colon carcinomas with the ERBB2 mutations harbored K-RAS mutations (Table 2). The incidence of ERBB2 mutation was significantly associated with K-RAS mutation in the colorectal carcinomas (Fisher’s exact test, P = 0.03). The detected K-RAS mutations in colorectal carcinomas with the three ERBB2 mutations consisted of two 35G>A (G12D) and one 35G>T (G12V).

To see whether the ERBB2 mutations were associated with amplification of ERBB2, we analyzed the tissues with the ERBB2 mutations by fluorescence in situ hybridization. However, we observed no ERBB2 amplification in the tissues (Fig. 3).

**Discussion**

The discovery of the ERBB2 kinase domain mutations in lung adenocarcinomas (11, 12) led us to analyze the possibility that other common human cancers might also possess the ERBB2 mutations. In this study, we found that ERBB2 mutations in the DNA sequences encoding the kinase domain occur in all of the three cancer types analyzed (gastric, breast, and colorectal carcinomas). The earlier reports have found an ERBB2 mutation in a gastric cancer, but none in either breast or colorectal cancer (11, 12). We observed a similar incidence of ERBB2 mutation (9 of 180, 5.0%) in gastric cancers compared with the previous data (1 of 20, 5.0%). In contrast with the previous data in breast and colorectal cancers, we found ERBB2 mutations in these cancers. The incidence of genetic alterations in some genes varies depending on race. For example, both EGFR and ERBB2 mutations in lung cancers were more frequent in patients from Japan than in those from the U.S. (3, 12). Our data also suggest that the incidence of ERBB2 mutations in breast and colorectal cancers might vary depending on the ethnicity of the cancer patients.

To date, there have been 11 types of ERBB2 kinase domain mutations, including 7 insertion/duplication mutations and 4 missense mutations (11, 12). In lung adenocarcinomas, all ERBB2 mutations but one were in-frame insertion/duplication mutations in a small stretch of exon 20 (amino acids 774-779), whereas all three mutations detected in other cancer types besides the lung cancer were missense mutations in exons 19, 20, and 21 (11, 12). Interestingly, in the present study, the ERBB2 mutations detected were all missense mutations except for one in-frame deletion/insertion, and the distribution of the exons with the mutations was not limited to exon 20 (Fig. 2).

Our data, together with the previous data, indicate that the type and location of ERBB2 kinase domain mutations in non–lung adenocarcinoma tumors may be different from those in lung adenocarcinoma, and suggest the possibility that the functional mechanism of the ERBB2 mutations in the development of gastric, breast, and colorectal carcinoma might be different from that of lung adenocarcinoma.

 Oncogene genes frequently occur at specific DNA sequences. In this study, four missense mutations showed an identical T to C transition at nucleotide 2264 (L755S) in unrelated patients (Table 1), and this amino acid sequence was mutated by a missense mutation in lung cancers (11). Another four mutations in this study affected the same amino acid (V777), which was within the region with frequent insertions/duplications of ERBB2 in the previous studies (11, 12). These data indicate that both L755 and V777 were affected in the frequently mutated sites, and suggest that these alterations could possibly be targeted therapeutically. It is interesting to compare the sites of ERBB2 mutations with those of the EGFR mutations. Two amino acids, V777 and L869, affected by the ERBB2 mutations in this study, are the equivalent amino acids, V769 and L861, respectively, affected in EGFR. Interestingly,}

**Table 2. Relationship of ERBB2 and K-RAS mutations in colorectal carcinomas**

<table>
<thead>
<tr>
<th>K-RAS mutation (+)</th>
<th>K-RAS mutation (−)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERBB2 mutation (+)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>ERBB2 mutation (−)</td>
<td>29</td>
<td>71</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>71</td>
</tr>
</tbody>
</table>

**Fig. 2.** Schematic organization of ERBB2 kinase domain and the locations of the mutations. The ERBB2 mutations detected in previous studies (refs. 11, 12; top) and in the current study (bottom) are marked by arrows. The descriptions for deletion or insertion are displayed, whereas the description for the missense mutation was marked only by the locations of the amino acids where the mutation occurred.
ERBB2 Mutation in Cancers

mutations of both V769 and L861 in EGFR have been reported in lung cancers (3, 6). Recurrent mutations of the amino acids at the same conserved sites within the EGFR family suggest that alterations of these amino acids may significantly change the function of the EGFR family protein in the affected cancer cells.

Mutant alleles of proto-oncogenes are considered dominant if they transform cells despite the presence of their normal alleles. ERBB2 gene, a proto-oncogene, has usually been mutated heterozygously in lung adenocarcinomas (12). However, Shigematsu et al. (12) reported two nonheterozygous ERBB2 mutations in lung adenocarcinomas. In the current study, we also detected one nonheterozygous L755_T759del, S760A mutation in a patient with breast carcinoma (case 43; Fig. 1A and D). However, the functional difference between monoallelic and biallelic alterations of the ERBB2 kinase domain remains unknown at this stage.

Molecules on the ERBB2 signaling pathway, including K-RAS, EGFR, PIK3CA, and BRAF are frequently mutated in human cancers and acts as oncogenic proteins (3–6, 20–22). To see whether the ERBB2 mutations accompanied these oncogenic mutations, we analyzed the mutations of these genes in tumors. Of these genes, we found the coincidence of ERBB2 and K-RAS mutations in colorectal cancers, suggesting that alterations of the ERBB2 signaling pathway both by ERBB2 and K-RAS mutations together contribute to the pathogenesis of colorectal cancers.

Whereas amplification of ERBB2 has frequently been detected in breast cancers, it is rarely observed in other common carcinomas, including gastric and colorectal cancers (23). We detected no ERBB2 amplification in cancers with ERBB2 mutations (Fig. 3), which was in agreement with a previous report (11) that revealed no association between ERBB2 mutation and ERBB2 amplification in lung cancers. These and our data suggest that ERBB2 kinase domain mutation might not be associated with the ERBB2 amplification in the cancers.

A central aim of cancer research has been to identify the mutated genes that are causally implicated in tumorigenesis. Mutations in cancer could be categorized either as functional alterations affecting key genes underlying the neoplastic process or as nonfunctional “passenger” changes (24). Modest incidence (2.9–5.0%) of the ERBB2 mutations, recurrent occurrence of the mutation in specific coding regions, and the similarity of some ERBB2 mutations with the EGFR mutations suggest that the ERBB2 mutations detected in this study might be functional alterations, but not passenger alterations.

Therapeutically, tyrosine kinases have become rational targets for cancer treatment. However, the patient selection is central to the successful development of the tyrosine kinase inhibitors. Because the presence of activating mutations seems to be an important determinant for the therapeutic effectiveness of kinase inhibitors, as in the case of EGFR kinase domain mutation in lung cancer patients to the EGFR kinase inhibitor Iressa (3–5), our data presented here may provide crucial information on the targeting of ERBB2 as a cancer therapy.

References
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