Somatic Mutations of the *HER2* Kinase Domain in Lung Adenocarcinomas

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Abstract

Mutations in the epidermal growth factor receptor gene (*EGFR*) in lung cancers predict for sensitivity to EGFR kinase inhibitors. *HER2* (also known as *NEU*, *EGFR2*, or *ERBB2*) is a member of the EGFR family of receptor tyrosine kinases and plays important roles in the pathogenesis of certain human cancers, and mutations have recently been reported in lung cancers. We sequenced the tyrosine kinase domain of *HER2* in 671 primary non–small cell lung cancers (NSCLC), 80 NSCLC cell lines, and 55 SCLCs and other neuroendocrine lung tumors as well as 85 other epithelial cancers (breast, bladder, prostate, and colorectal cancers) and compared the mutational status with clinicopathologic features and the presence of *EGFR* or *KRAS* mutations. *HER2* mutations were present in 1.6% (11 of 671) of NSCLC and were absent in other types of cancers. Only one adenocarcinoma cell line (NCI-H1781) had a mutation. All *HER2* mutations were in-frame insertions in exon 20 and target the identical corresponding region as did *EGFR* insertions. *HER2* mutations were significantly more frequent in never smokers (3.2%, 8 of 248; $P = 0.02$) and adenocarcinoma histology (2.8%, 11 of 394; $P = 0.003$). In 394 adenocarcinoma cases, *HER2* mutations preferentially targeted Oriental ethnicity (3.9%) compared with other ethnicities (0.7%), female gender (3.6%) compared with male gender (1.9%) and never smokers (4.1%) compared with smokers (1.4%). Mutations in *EGFR*, *HER2*, and *KRAS* genes were never present together in individual tumors and cell lines. The remarkable similarities of mutations in *EGFR* and *HER2* genes involving tumor type and subtype, mutation type, gene location, and specific patient subpopulations targeted are unprecedented and suggest similar etiologic factors. *EGFR*, *HER2*, and *KRAS* mutations are mutually exclusive, suggesting different pathways to lung cancer in smokers and never smokers. (Cancer Res 2005; 65(5): 1642-6)

Introduction

Activating mutations of protein kinases contribute to the development of human cancers (1) and have led to the development of kinase inhibitors that target these oncogenic forms (2). The inhibitors include imatinib mesylate (Gleevec, STI571), a small molecule inhibitor of the Abl and KIT tyrosine kinases (TK) for patients with chronic myelogenous leukemia or gastrointestinal stromal tumors (3, 4), and gefitinib (Iressa, ZD1839) and erlotinib (Tarceva, OSI-774) inhibitors of epidermal growth factor receptor (EGFR) which are used for the treatment of non–small cell lung cancer (NSCLC). Recent reports of mutations in the TK domain of *EGFR* have generated considerable interest because they predict for sensitivity to gefitinib or erlotinib (5–7). The promising results of these TK inhibitors invoke the concept of "oncogene addiction," which hypothesizes that cancer cells are both transformed and physiologically dependent on activated oncogenes for their survival (8, 9). Thus, oncogenic activation of protein kinases including EGFR family members should be targets for cancer therapies.

HER2 (also known as *NEU*, *EGFR2*, or *ERBB2*) is one of the members of the EGFR family, which includes *EGFR* (or *ERBB1*), *EGFR3* (or *HER3*/*ERBB3*) and *EGFR4* (or *HER4*/*ERBB4*). Although the genes contain extracellular, transmembrane and intarcellular domains, the regions of greatest homology are the kinase domains contained within an intracellular domain. However, the genes have distinct properties: HER2 has strong kinase activity but has no identified ligand and ERBB3 lacks kinase activity due to substitutions in critical TK domain residues (10). All are capable of forming homodimers (with the possible exception of ERBB3) and heterodimers. *EGFR* and *HER2* are dysregulated in many human cancers and play important roles in cancer development and progression (11). Overexpression of *HER2* with amplification is found in a subset of breast and ovarian cancers and correlates with poor prognosis (12, 13). In lung cancers, overexpression of *HER2* has been reported in about 20% (14–17) whereas gene amplification occurs less frequently than in breast cancers. Trastuzumab (Herceptin), a humanized monoclonal antibody that binds the extracellular domain of HER2, is effective for HER2 overexpressing breast cancer patients when used with other cytotoxic agents (18). By contrast, clinical trials using Trastuzumab in NSCLC patients have reported modest or disappointing clinical benefits (19–21). Recently, mutations of *HER2* were reported in lung adenocarcinomas and offer the potential of additional therapy targeted at the altered protein (22). In this report, we searched for mutations of *HER2* in a large number of primary lung tumors from four countries (Japan, Taiwan, the United States, and Australia). Because mutations in the *EGFR* gene target adenocarcinoma histology, female gender, never smoking status, and Oriental ethnicity (6, 7, 23), we

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determined whether there was a relationship between *HER2* mutations and some or all of these factors. In addition, we correlated the mutation status of *HER2*, *EGFR*, and *KRAS* genes.

Materials and Methods

Tumor Samples. A total of 671 primary NSCLCs were obtained from patients undergoing curative intent surgical resections from four countries (Table 1). All cases from Japan and Taiwan were of Oriental ethnicity. United States cases comprised 137 Caucasian, 8 Hispanic, 6 African American, and 4 Oriental (ethnicity was not available for two cases). Australian cases were all Caucasian except for one Oriental. A total of 36 neuroendocrine lung tumors including bronchial carcinoids (n = 25) and large cell neuroendocrine carcinomas (n = 5) from the United States and SCLC (n = 6) from Japan were also studied. Eighty-five carcinomas arising at other sites (breast, n = 28; bladder, n = 15; prostate, n = 14; and colorectal cancer, n = 28) were obtained from the hospitals affiliated with the University of Texas Southwestern Medical Center. Institutional Review Board permission and informed consent were obtained at each collection site. Clinical information including gender, age, histology, clinical stage, and smoking history were available. Clinical staging was based on the revised International System for Staging Lung Cancer (24). We also studied 80 NSCLC cell lines established by us (25) except for NCI-H3255 which was initiated by and obtained from Dr. Bruce Johnson (Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Boston, MA; ref. 26). The list of cell lines studied and their mutational status may be obtained from the senior author.

DNA Extraction and Sequencing of *HER2*, *EGFR*, and *KRAS*. Genomic DNA was obtained from primary tumors and cell lines by overnight digestion with SDS and proteinase K (Life Technologies, Inc., Rockville, MD) at 37C followed by standard phenol-chloroform (1:1) extraction and ethanol precipitation.

The intron-based PCR primer sequences for seven examined exons of the entire *HER2* TK domains were as follows (forward and reverse, respectively): Exon 18 (5′-GTGAAGTCCTCCCAGCCCGC-3′ and 5′-CTCCCATCAGAACTGCCGACC-3′), Exon 19 (5′-TGGAGGACAAGTAATGATCTCCTGG-3′ and 5′-AAGAGAGACCAGAGCCCAGACCTG-3′), Exon 20 (5′-GCCATGGCTGTGGTTTGTGATGG-3′ and 5′-ATCCTAGCCCCTTGTGGACATAGG-3′), Exon 21 (5′-GGACTCTTGCTGGGCATGTGG-3′ and 5′-CCACTCAGAGTTCTCCCATGG-3′), Exon 22 (5′-CCATGGGAGAACTCTGAGTGG-3′ and 5′-TCCCTTCACATGCTGAGGTGG-3′), Exon 23 (5′-AGACTCCTGAGCAGAACCTCTG-3′ and 5′ -AGCCAGCACAGCTCAGCCAC-3′), and Exon 24 (5′-ACTGTCTAGACCAGACTGGAGG-3′ and 5′-GAGGGTGCTCTTAGCCACAGG-3′). All PCRs were carried out in 25-μL volume containing 100 ng of genomic DNA using HotStarTaq DNA polymerase (QIAGEN Inc., Valencia, CA). DNA was amplified for 32 to 34 cycles at 95°C for 30 seconds, 62°C to 68°C for 30 seconds, and 72°C for 30 seconds followed by 7 minutes extension at 72°C. All PCR products were incubated using exonuclease I and shrimp alkaline phosphatase (Amersham Biosciences Co., Piscataway, NJ) and sequenced directly using Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer Co., Foster City, CA). All sequence variants were confirmed by independent PCR amplifications and sequenced in both directions.

EGFR (exons 18-21) and *KRAS* (codons 12 and 13) mutation status were determined using the intron-based PCR primers as described previously (23). The *EGFR* and *RAS* data from some of these samples have been reported elsewhere (23).

Statistical Analyses. Fisher's exact tests were used to assess the relation between *HER2* mutations and each factor. All statistical tests were two-sided and $P < 0.05$ were considered statistically significant.

Results and Discussion

Preliminary sequencing of the entire *HER2* TK domain (exons 18-24) in 96 unselected NSCLC samples or sequencing of the first four exons of the TK domain (exons 18-21) in which *EGFR* mutations are limited, in 200 unselected NSCLC samples, and 28 breast carcinoma samples indicated that mutations were limited to exon 20. Because one missense mutation in exon 19 was reported previously in a NSCLC tumor (22), further analyses were limited to these two exons.

Mutations were limited to NSCLC and were absent in 36 neuroendocrine lung tumors (SCLC, large cell neuroendocrine carcinoma, and bronchial carcinoids) and tumors from other sites. A total of 11 (1.6%) mutations were detected in 671 primary NSCLC cases and one (1.3%) mutation was found in 80 NSCLC cell lines (Table 1). All mutations were in-frame duplications/insertions. Corresponding nonmalignant tissues was available from 9 of 11 mutant cases, and the *HER2* mutations were confirmed as being somatic in origin. No missense mutations were found. According to the electropherograms (Fig. 1), most mutations were heterozygous, whereas NCI-H1781 (Fig. 1*B*) and Japan 79 (Fig. 1*D*) seemed to be homozygous (no wild sequence was detected). These results indicated that allelic imbalance due to the loss of wild allele or selective amplification of the mutant allele occurred in these samples.

Table 1. KRAS, EGFR, and HER2 mutation in resected NSCLC

Country (n)	KRAS	EGFR	HER2						
	Mutation (%)	Mutation (%)	Mutation (%)	Gender (n)	Mutation (%)	Smoking (n)	Mutation (%)	Histology* (n)	Mutation (%)
Japan (269)	17 (6)	70 (26)	8 (3.0)	Male (193)	2 (1.0)	NS (77)	5 (6.5)	Adeno. (157)	8 (5.1)
				Female (76)	6 (7.9)	S (192)	3 (1.6)	Others (112)	0
Taiwan (145)	5 (3)	52 (36)	2 (1.4)	Male (71)	1 (1.4)	NS (106)	2 (1.8)	Adeno. (94)	2 (2.1)
				Female (74)	1 (1.4)	S (39)	0	Others (51)	0
United States (157)	19 (12)	15 (10)	0	Male (85)	0	NS (40)	0	Adeno. (97)	0
				Female (72)	0	S (117)	0	Others (60)	0
Australia (100)	13 (13)	12 (12)	1 (1.0)	Male (64)	1 (1.6)	NS (25)	1 (4.0)	Adeno. (46)	1 (2.2)
				Female (36)	0	S (75)	0	Others (54)	0
Total (671)	54 (8)	149 (22)	11 (1.6)	Male (413)	4 (1.0)	NS (248)	8 (3.2)	Adeno. (394)	11 (2.8)
				Female (258)	7 (2.7)	S (423)	3 (0.7)	Others (277)	0

Abbreviations: NS, never-smoker; S, smoker; Adeno., adenocarcinoma.
*Others include squamous cell, adenosquamous cell, and large cell carcinoma.

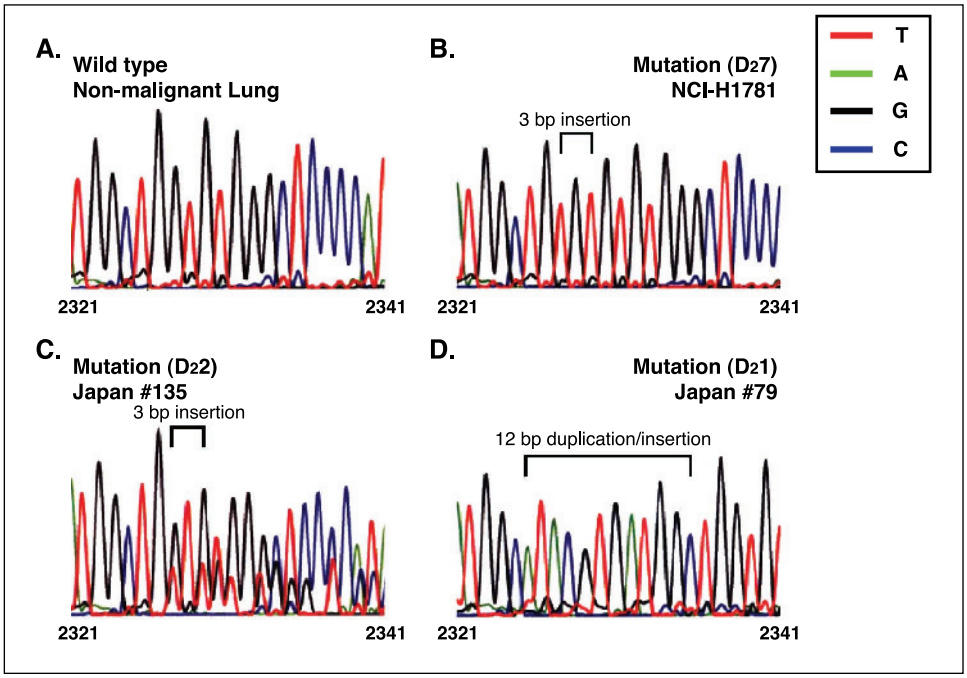

FIgure 1. Electropherograms demonstrating mutational patterns. *A,* wild-type sequence in nonmalignant lung tissue. *B,* 3-bp insertion ($D_2$7: homozygous) in NCI-H1781. *C,* 3-bp insertion ($D_2$2: heterozygous) in primary tumor. *D,* 12-bp duplication/insertion ($D_2$1: homozygous) in primary tumor.

All 11 *HER2* mutant cases had adenocarcinoma histology and seven cases occurred in female patients (Table 2). Of the 11 mutant cases, nine of the subjects were never smokers or very light smoker (0.5 pack year history). By contrast, 420 (64%) of the 660 subjects whose tumors lacked mutations were smokers. *HER2* mutations preferentially targeted Oriental countries (Japan and Taiwan; 2.4%) compared with Western countries (the United States and Australia; 0.3%) and female gender (2.7%) compared with male gender (1.0%); however, these differences were not statistically significant (P = 0.06 and 0.12 respectively). They occurred more frequently in never smoker (3.2%) than in smoker (0.7%) and in adenocarcinoma (2.8%) than in other histologies (0%; P = 0.02 and 0.004, respectively; Table 1). In 394 adenocarcinoma cases, *HER2* mutations preferentially targeted Oriental ethnicity (3.9%) compared with other ethnicities (0.7%), female gender (3.6%) compared with male gender (1.9%) and never smokers (4.1%) compared with smokers (1.4%). These findings are similar to the subpopulations of lung cancers in whom *EGFR* mutations occur as previously shown by us and others (7, 23). In the selected subgroup of Oriental female never smokers with adenocarcinoma histology, the frequency of *EGFR* mutations was 61% and of *HER2* mutations was 4.3%. These results suggest that similar genetic factors and possibly carcinogen(s) or other

Table 2. HER2 mutation in lung cancer

Origin/ country	Sample no.	Sex	Age	Histology	Smoking (pack-years)	Stage	Nucleotide	Amino acid	Designation*
Primary tumor									
Japan	60	F	74	Adenocarcinoma	NS	I	2325-2336 ins ATACGTGATGGC	YVMA 776-779 ins	$D_2$1
	79	F	58	Adenocarcinoma	NS	I	2325-2336 ins ATACGTGATGGC	YVMA 776-779 ins	$D_2$1
	135	F	58	Adenocarcinoma	S (0.5)	I	2327-2329 ins TTT	G776V, Cins	$D_2$2
	153	F	66	Adenocarcinoma	NS	III	2327-2329 ins TTT, 2326 G>C	G776L, Cins	$D_2$3
	154	F	52	Adenocarcinoma	S (5)	III	2340-2348 ins GGGCTCCCC	GSP 781-783 ins	$D_2$4
	189	M	63	Adenocarcinoma	S (40)	I	2325-2336 ins ATACGTTGATGGC	YVMA 776-779 ins	$D_2$1
	254	F	68	Adenocarcinoma	NS	I	2341-2349 ins GGCTCCCCA	GSP 781-783 ins	$D_2$5
	276	M	58	Adenocarcinoma	NS	I	2325-2336 ins ATACGTTGATGGC	YVMA 776-779 ins	$D_2$1
Taiwan	11	M	72	Adenocarcinoma	NS	II	2327-2329 ins TTT	G776V, Cins	$D_2$2
	438	F	76	Adenocarcinoma	NS	I	2325-2336 ins ATACGTGATGGC	YVMA 776-779 ins	$D_2$6
Australia	478	M	83	Adenocarcinoma	NS	I	2326-2337 ins TACGTGATGGCT	YVMA 776-779 ins	$D_2$6
Cell line	NCI-H1781	F	66	Adenocarcinoma	S (60)	III	2327-2329 ins TGT	G776V, Cins	$D_2$7

Abbreviations: M, male; F, female; NS, never smoker; S, smoker; ins, insertion.
*Designation terminology: We had previously assigned designations to *EGFR* mutations by mutation type followed by a number for mutation variation (24). D, duplications/insertions. To distinguish *EGFR* gene duplications/insertions from those in the *HER2* gene, we assigned the subscript D_2 for the former.

environmental factor(s) affect the occurrence of mutations in both genes.

All of the *HER2* mutations were in-frame duplications/insertions in a small stretch of exon 20 (Fig. 2; Table 2). Whereas they were of four different types, a 12-bp duplication/ insertion coding for the amino acids YVMA at codon 776 was the major pattern (6 of 11 mutations). We also detected a 9-bp duplication/insertion (2 of 11 mutations) and three individual base pair *de novo* insertions. Interestingly, *HER2* mutations target the identical corresponding nine-codon region in exon 20 as did *EGFR* duplications/insertions (Fig. 2). *EGFR* mutations target important structures around the ATP binding cleft which is also the docking site of the TK inhibitors (9, 23), including the phosphate binding loop, the αC-helix, and the activation loop. In-frame duplications/insertions of *EGFR* occur at the COOH-terminal end of αC-helix, and postulated by us (9) presumably result in configurational changes causing a shift of the helical axis, narrowing the ATP binding cleft and resulting in both increased gene activation and TK inhibitor sensitivity.

It is of interest to compare our findings with those previously reported (22). Stephens et al. found two types of duplications/ insertions in exon 20 (total of four cases). Whereas these were different from the ones we found (Fig. 2), all duplications/ insertions from both studies targeted the same eight-codon region (codons 774-781). All of the mutations they identified in lung cancers were in adenocarcinomas (similar to our findings). However, four of the five mutations in their cases were in current or former smokers, in contrast to our findings that the mutations targeted never smokers. Of interest, in their series, *EGFR* mutations in lung adenocarcinomas were less frequent than *HER2* mutations (although we are not informed as to which exons were examined). However, Stephens et al. do not present any detailed data regarding gender, histology, ethnicity, smoking status, or even the geographic location of their lung cancers. Thus, a detailed comparison with our findings is not possible.

We previously reported that *EGFR* and *KRAS* mutations do not occur simultaneously (23). Ras/Raf/mitogen-activated protein kinase signaling is one of the important EGFR downstream pathways. The additional cases analyzed in the present study confirm and extend our previous findings. *KRAS* mutations were detected in 54 (8%) and *EGFR* mutations were detected in

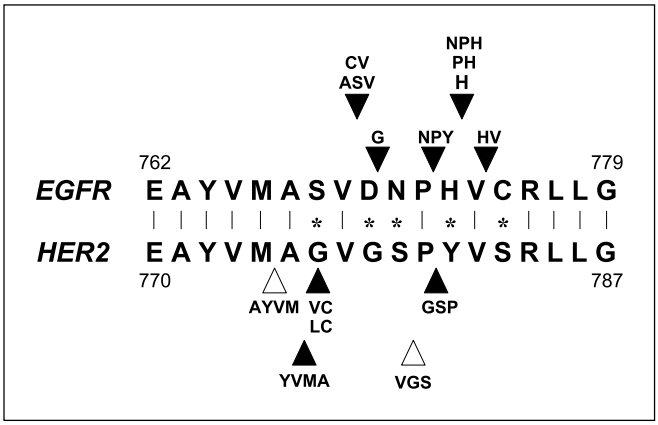

Figure 2. Amino acid alignments of the tyrosine kinase domain in EGFR and HER2. ▲ and ▼, duplications/insertions in this study (for *HER2*) or previously reported by us for *EGFR* (23). △, *HER2* duplications/insertions described by Stephens et al. (22). *, nonconserved amino acid.

Table 3. EGFR, HER2, and KRAS mutation status in NSCLC cell lines

No. cell lines	Mutation status		
	EGFR	HER2	KRAS
8 (10%)	Mu	Wt	Wt
1 (1%)	Wt	Mu	Wt
20 (25%)	Wt	Wt	Mu
51 (64%)	Wt	Wt	Wt
Total 80 (100%)			

Abbreviations: Mu, mutant type; Wt, wild type.

149 (22%) of 671 NSCLC patients (Table 1). In 80 NSCLC cell lines, *KRAS* mutations were detected in 20 (25%) and *EGFR* mutations were detected in eight (10%; Table 3). Mutations in more than one of these genes were never found simultaneously in the same tumor, suggesting that activation of either *EGFR*, *HER2*, or oncogenic *KRAS* is sufficient for lung carcinogenesis. Of interest, HER2 is the preferential heterodimer partner for EGFR, and interactions between family members may play a role in lung cancer pathogenesis. Previously, we hypothesized that at least two distinct molecular pathways are involved in the pathogenesis of lung adenocarcinomas, one involving *EGFR* mutations in never smokers and the other involving oncogenic *KRAS* mutations in smokers (9). *HER2* mutations may contribute to lung adenocarcinoma pathogenesis in never smokers. However, only 51% of adenocarcinomas in the present study had mutations in any one of the three genes, indicating a role for other as yet unknown genetic or epigenetic changes. Of interest, in the highly selected subgroup of Oriental female never smokers with adenocarcinoma histology lacking *EGFR* mutations, the frequency of *HER2* mutations was 11%.

Oncogenic mutations activate kinases by disrupting the autoinhibitory mechanisms that normally stabilize their inactive forms (1). They target structures around the ATP binding cleft that are involved in phosphorylation events. These structures include the phosphate binding and activation loops. Of interest, very different kinases, such as the receptor tyrosine kinase *PDGFR* family and the intracellular serine/threonine kinase *BRAF* share similar oncogenic hotspots (1). Deletions and duplications/ insertions on either side of the αC-helix are also characteristic of *EGFR* mutations, and we have postulated that such mutations alter the angle of the ATP binding cleft, resulting in greater activity (9). Of interest, with one exception, all of the *HER2* mutations in lung cancers described by us and by Stephens et al. (22) have been in-frame duplications/insertions targeting a region of eight codons in exon 20 on the COOH-terminal side of the αC-helix. With one exception they occur adjacent to or replaced nonconserved residues (Fig. 2). Whereas the activating function of these specific mutations has not been clarified, the remarkable similarities between the mutations in *EGFR* and *HER2* suggest that they are functional. However, the function of the very rare, often unique, point mutations found in NSCLC and other cancers is less certain.

In summary, we found a relatively modest frequency of somatic *HER2* gene mutations limited to the adenocarcinoma subtype of lung cancer. The mutations targeted never or light smokers,

Oriental ethnicity and female gender. The remarkable similarities of mutations in *EGFR* and *HER2* genes involving tumor type and subtype, mutation type, gene location, and specific patient subpopulations targeted are unprecedented in molecular medicine. These finding suggest the necessity of epidemiologic studies focused on finding a common underlying etiology. *HER2* mutant cell line NCI-H1781 is resistant *in vitro* to gefitinib (26), which preferentially inhibits the TK activity of *EGFR*. The identification of NCI-H1781 with its *HER2* mutation provides an important new resource for preclinical therapeutic studies looking a *HER2* targeted agents. However, broad spectrum TK inhibitors may also offer the advantage of simultaneously targeting multiple members of the *EGFR* family thereby interfering with the cooperation that exists between receptors (27).

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