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Microsatellite Instability at Selected Tetranucleotide Repeats Is Associated with p53 Mutations in Non-Small Cell Lung Cancer[1]

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ABSTRACT

Microsatellite alterations are useful clonal markers for the early detection of cancer. An increase in microsatellite instability has been observed at certain tetranucleotide repeat markers ($AAAG_n$) in lung, head and neck, and bladder cancer. However, the genetic mechanism underlying these elevated microsatellite alterations at selected tetranucleotide repeat (EMAST) tumors is still unknown. The *p53* gene plays an important role in maintaining genome integrity by repairing damaged DNA. Therefore, we tested 88 non-small cell lung cancers with a panel of 13 microsatellite markers previously shown to exhibit frequent instability and also performed *p53* sequence analysis in these tumors. Thirty-one of these 88 cancers (35%) demonstrated a novel allele [EMAST(+)] in ≥1 of these 13 microsatellite markers. *p53* mutations were detected in 50 of 88 (57%) cancers and were significantly ($P = 0.001$) more common in EMAST(+) tumors (25 of 31; 81%) than in EMAST(−) tumors (25 of 57; 44%). Among squamous cell cancers, *p53* mutations were detected significantly ($P = 0.04$) more frequently in EMAST(+) tumors (17 of 19; 89%) than in EMAST(−) tumors (10 of 18; 55%). Similarly, among primary adenocarcinomas, *p53* mutations were present in 67% of the EMAST(+) tumors and in 35% of EMAST(−) adenocarcinomas. None of the 31 EMAST(+) tumors demonstrated high frequency microsatellite instability when examined with a reference panel of five mono- and dinucleotide markers. Primary lung cancers with microsatellite alterations at selected tetranucleotide repeats have a high frequency of *p53* mutations and do not display a phenotype consistent with defects in mismatch repair.

INTRODUCTION

MSI[3] was initially identified in colorectal cancer and was immediately clinically significant because of its association with HNPCC (1). MSI is thought to be caused by a failure of the mismatch repair system to repair errors that occur during DNA replication (1). Several mismatch repair genes have been identified (*hMLH1, hMSH2, hPMS1, hPMS2,* and *hMSH6*), and their inactivation has been well-characterized in HNPCC and occasionally in other noncolonic tumors with MSI (1, 2). A genetically and clinically distinct form of MSI has also been described in which microsatellite alterations are present at selected tri- and tetranucleotide repeats in noncolonic, non-HNPCC tumors (1). In these tumors, larger repeats are more commonly altered than smaller repeats. This finding is in marked contrast to observations in HNPCC and most sporadic gastric and endometrial tumors, which display an elevated level of instability at mono- and dinucleotide repeats (1). This distinct subtype of MSI has been termed EMAST (1). These observations in NSCLC have recently been expanded and described in greater detail.[4]

Although the genetic basis for MSI associated with defective mismatch repair is being increasingly clarified, the mechanism underlying microsatellite alterations in EMAST tumors has yet to be identified (1). The lack of overlap between genome wide MSI and EMAST(+) tumors suggests that a nonmismatch repair pathway might be involved (1). The p53 protein is a significant component of the cellular response to DNA damage (3). Accumulation of p53 protein leads to cell cycle arrest to prevent the replication of damaged DNA, and if the DNA is not repaired, it can lead to apoptosis in cells with severe DNA damage (3, 4). The *p53* gene is frequently mutated in NSCLC, and similar to EMAST(+) status, these mutations occur more commonly in squamous cell lung cancer than in adenocarcinoma of the lung (5). Therefore, we examined the relationship between *p53* gene mutations and EMAST in NSCLC.

MATERIALS AND METHODS

Sample Collection. Primary tumor, normal lung, and blood were collected from 88 patients undergoing surgical resection of NSCLC at The Johns Hopkins Hospital or the Johns Hopkins Bayview Medical Center. Pathological stage was determined using the revised International System for Staging Lung Cancer (6). Lymphocytes were collected from blood and used a source of normal DNA. Tumor samples and normal lung tissue were promptly frozen at −80°C after initial gross pathological examination. The Joint Committee on Clinical Investigation of The Johns Hopkins School of Medicine approved this research protocol, and written informed consent was obtained from all patients.

Portions of the primary tumor were cut into 7-μm sections, stained with H&E, and examined by light microscopy. Additional 12-μm sections were cut and placed in a mixture of 1% SDS and proteinase K at 48°C overnight. Tumors with a low neoplastic cellularity (<70%) were further microdissected to remove contaminating normal cells. DNA was extracted with phenol/chloroform and precipitated with ethanol.

Microsatellite Analysis. Microsatellite analysis was performed on all 88 tumors using a panel of 13 markers (*L17686, D20S82, UT5320, D8S321, L17835, D20S85, UT5307, D9S242, G29028, D11S488, ACTβ2, G08460,* and *CSFIR1*) previously shown to demonstrate frequent microsatellite alterations (expansion or deletion of a repeat unit) at tetranucleotide repeats in NSCLC (5). Results of microsatellite analysis using these markers on 50 of these tumors have been reported previously (5). In addition, tumors harboring microsatellite alterations with the 13-marker panel were also analyzed using the Bethesda Consensus Conference reference panel of five mono- and dinucleotide markers (*BAT-25, BAT-26, D2S123, D5S346, D17S250*) for determining MSI in colorectal cancer (no similar panel has been selected to date for NSCLC; Ref. 1). Using this reference panel, MSI-H tumors are defined as having instability in two or more markers, low frequency MSI tumors are defined as having instability in one marker, and microsatellite stable tumors demonstrate no instability (1). These reference markers have been shown to identify those tumors with defective mismatch repair, primarily in the two major mismatch repair genes, *hMSH2* and *hMLH1* (1). Primers were obtained from Research Genetics (Huntsville, AL) or synthesized based on sequences in the Genome Database. One

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[3] The abbreviations used are: MSI, microsatellite instability; HNPCC, hereditary nonpolyposis colon cancer; NSCLC, non-small cell lung cancer; EMAST, elevated microsatellite alterations at selected tetranucleotide repeats; MSI-H, MSI-high frequency.

[4] J. Jen and D. Sidransky, submitted for publication.

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marker from each primer pair was labeled with T4 polynucleotide kinase (New England Biolabs). PCR amplification was performed in separate reactions with 60 ng of DNA isolated from the tumor, and control non-neoplastic DNA as described above. Products were separated in 8% denaturing urea-polyacrylamide-formamide gels followed by autoradiography.

p53 Sequencing. Mutation analysis of the *p53* gene was performed on all 88 lung cancers by both direct dideoxy nucleotide sequencing and using the GeneChip *p53* assay (Affymetrix Inc., Santa Clara, CA) as reported previously (7). A 1.8-kb fragment of the *p53* gene (exons 5–9) was amplified from primary tumor DNA in all 88 patients by PCR (8, 9). The PCR products were purified and sequenced directly using cycle sequencing (Amplicycle sequencing kit, Perkin-Elmer, Branchburg, NJ), and the products of the sequencing reactions were then separated by electrophoresis in 8 M urea and 6% polyacrylamide gels, fixed, and exposed to film. In addition, exons 2–11 of the *p53* gene from all 88 tumors were sequenced using the GeneChip p53 assay as described (7).

Statistical Analysis. Comparisons between groups were performed using the Fisher's exact test (two-tailed).

RESULTS

Pathological Characteristics. Pathological characteristics from 88 patients undergoing pulmonary resection for NSCLC were reviewed. Forty-four patients had stage I disease (26 stage IA and 18 stage IB), 24 patients had stage II NSCLC (3 stage IIa and 21 stage IIb), and 20 patients had stage III disease. Mean tumor size was 3.8 cm. The cell type of the 88 NSCLCs included squamous cell cancer ($n = 37$), adenocarcinoma ($n = 44$), adenosquamous carcinoma ($n = 1$), large cell carcinoma ($n = 4$), and poorly differentiated NSCLC ($n = 2$).

Microsatellite Alterations. Thirty-one of these 88 cancers (35%) demonstrated a novel allele [EMAST(+)] with at least 1 of 13 microsatellite markers (Fig. 1). Twenty-two of 31 EMAST(+) tumors had MSI at 1 marker, whereas 9 of the EMAST(+) tumors demonstrated microsatellite alterations at ≥2 of 13 markers. These microsatellite "shifts" were seen in 12 tumors (14%) at *L17686*, 7 tumors (8%) at *D20S82*, 6 (7%) tumors at *UT5320*, 4 (4%) tumors at *8S321*, 3 (3%) tumors at *L17835* and *D20S85*, 2 (2%) tumors at two markers (*UT5307* and *D9S242*), and in 1 tumor (1%) at four markers (*G29028*, *D11S488*, *ACTβ2*, and *G08460*). Squamous cell cancers were significantly more likely to be EMAST(+) than adenocarcinoma of the lung (51% *versus* 20%; $P = 0.006$). No significant association between patient age, race, gender, tumor size, tumor stage, and tumor differentiation and EMAST status was observed.

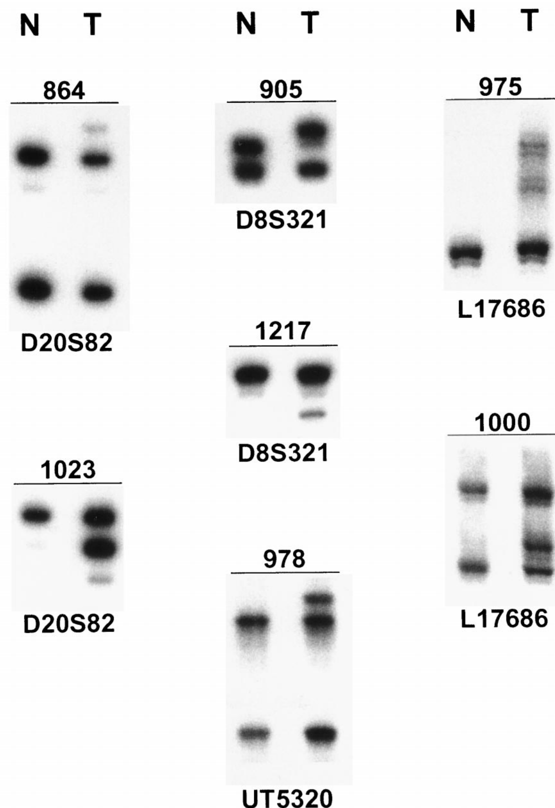

Fig. 1. Identification of MSI in NSCLC. MSI (insertion or deletion of repeat units) in tumor (*T*) compared to nonneoplastic DNA (*N*).

Table 1 *Frequency of p53 mutations in EMAST(+) and EMAST(−) NSCLC*

Cell type	n^a	% with *p53* mutations	*P*
Squamous cell cancer			
EMAST(−)	18	55	0.04
EMAST(+)	19	89	
Total	37	73	
Adenocarcinoma			
EMAST(−)	35	35	0.15
EMAST(+)	19	67	
Total	44	41	
All NSCLC[b]			
EMAST(−)	57	44	0.001
EMAST(+)	31	81	
Total	88	57	

[a] *n*, number of patients.
[b] Also includes seven large cell or poorly differentiated tumors.

All 31 EMAST(+) tumors were examined with a reference panel of five mono- and dinucleotide markers used for determining MSI. Only 1 of 31 EMAST(+) tumors (3%) had instability at a single marker (low frequency microsatellite instability), and the remaining 30 tumors were microsatellite stable.

EMAST and p53. p53 mutations were detected in 50 of 88 (57%) patients with NSCLC. Mutations of the *p53* gene were found significantly ($P = 0.006$) more often in squamous cell cancer [27 of 37 (73%)] than in adenocarcinoma [18 of 44 (41%)]. Thirty-three (66%) of the p53 mutant tumors contained a missense mutation, eight (16%) contained a nonsense mutation, five (10%) contained a deletion, and four (8%) contained a splice site mutation.

The relationship between EMAST positivity and mutation of the *p53* gene is shown in Table 1. *p53* mutations were significantly more common in EMAST(+) than in EMAST(−) NSCLC (81% *versus* 44%; $P = 0.001$). Among squamous cell cancers, *p53* mutations were also significantly ($P = 0.04$) more common in EMAST(+) than in EMAST(−) tumors (89% *versus* 55%). A similar trend was observed with adenocarcinoma of the lung, but this difference did not reach statistical significance (67% *versus* 35%; $P = 0.15$). Seven of nine tumors (78%) with MSI at two or more markers contained a *p53* mutation.

Eighty % (4 of 5) of the tumors containing a deletion in the *p53* gene were EMAST(+), whereas only 47% (21 of 45) of tumors containing a single bp mutation were also EMAST(+). Only 16% of the tumors with wild-type *p53* were EMAST(+).

DISCUSSION

Although widespread genome wide MSI is rare in NSCLC, microsatellite alterations at selected tetranucleotide repeats are not uncommon in primary lung cancer. Interestingly, these microsatellite alterations were more common in squamous cell cancer than in adenocarcinoma of the lung. Furthermore, EMAST(+) tumors were significantly more likely to harbor a *p53* mutation than EMAST(−) cancers.

Microsatellite alterations have become useful clonal markers for

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the early detection of cancer. The generation of novel alleles through the insertion or deletion of a short tandem repeat creates a new DNA marker present only in neoplastic and not in nonneoplastic cells. These markers are easier to detect in clinical samples than subtle changes in loss of heterozygosity and have shown promise in the early detection of bladder, lung, and head and neck cancers in preliminary studies (5, 10–15). The detection of novel microsatellite alleles in the urine has been used to identify both primary and recurrent bladder cancer (11, 12). In several cases, these microsatellite alterations have preceded any clinically detectable (urine cytology, cystoscopy) signs of cancer. In addition, tumor-specific microsatellite alterations have been detected in the bronchoalveolar lavage fluid, sputum, and plasma of patients with lung cancer, suggesting that these alterations may also be potential markers for the early detection of this common malignancy (5, 14, 15).

The association between gene alterations and MSI has been evaluated previously in several tumor types. Frameshift mutations within mononucleotide repeat sequences in several genes (*TGFβ-II*, *BAX*, *IGFRII*, *E2F-4*, and *TCF-4*) have been identified in MSI-H colon cancer (16–20). The human *BAX* gene, which promotes apoptosis, is inactivated by frameshift mutation in ~50% of HNPCC syndrome colon cancers (17, 19). Rampino *et al.* (17) have suggested that *BAX* gene inactivation downstream from *p53* in the apoptotic pathway would eliminate the selective pressure for *p53* gene mutations in colorectal tumorigenesis. Indeed, the incidence of *p53* gene mutations is lower in MSI-H colon cancer than in sporadic colon cancer (17, 19).

In contrast to colon cancer, a strong correlation between MSI and *p53* mutations has been reported in ovarian cancer (21). MSI was more frequent in tumors containing an insertion or deletion mutation than in tumors with a missense mutation, and 50% of the deletions occurred at iterated sequences or direct repeats (16). Sood *et al.* (21) concluded that the pattern of *p53* mutations (insertions/deletions at iterated bases) observed in ovarian cancer was consistent with DNA strand slippage during replication, and therefore, *p53* mutations were likely caused by a generalized genomic instability rather than being the cause of genomic instability. In contrast to these findings, no association between MSI and p53 mutations has been found in gastric cancer, and it was also not observed in the present series of NSCLCs using the Bethesda Conference criteria for defining MSI (22, 23).

MSI attributable to a defect in a DNA repair pathway distinct from mismatch repair has been hypothesized but remains undiscovered. The p53 protein plays a central role in the repair of genetically damaged cells. In response to DNA damage, p53 protein levels rapidly increase by a posttranscriptional mechanism (24). The induction of *p53* also results in the transcriptional activation of other cell cycle regulatory genes, such as $p21^{\mathrm{WAF\text{-}1}}$ leading to arrest of the cell cycle at the G_1-S checkpoint (24). Loss of functional p53 protein leads to a failure of cell cycle arrest and genomic instability, as evidenced by inopportune gene amplification, aneuploidy, and/or chromosome loss through DNA strand breakage and rejoining (4, 24, 25). The inability to delay cell division and allow adequate DNA repair to take place increases the probability that DNA damage will remain uncorrected during DNA replication, leading to the proliferation of genetically damaged cells (4). The failure to repair deletions or mutations in critical tumor suppressor genes can provide affected clones with a growth advantage. In this study, microsatellite alterations at selected tetranucleotide repeat markers were more commonly present in tumors containing a *p53* mutation. These clonal alterations in the noncoding DNA at the microsatellite markers examined are unlikely to provide any growth advantage to the tumor and may simply reflect the defective DNA repair capability of *p53* mutant cells. However, an increased mutation rate at tri- or tetranucleotide repeat sequences within genes regulating cell growth may provide a growth advantage to *p53* mutant cells. Germ-line studies have documented a higher background rate of instability in larger (tri- or tetranucleotide) microsatellites, which perhaps explains why these particular microsatellites are commonly altered in *p53* mutant primary tumors (26, 27).

Microsatellite alterations at selected tetranucleotide markers are common in non-small cell lung, head and neck, and bladder cancer. This study suggests that the underlying mechanism leading to EMAST(+) tumors differs from MSI attributable to defective mismatch repair and is more likely related to abrogation of a *p53*-dependent repair pathway. A better understanding of this pathway leading to the EMAST phenotype in primary tumors may help further define the role of this diagnostic strategy in cancer screening. The widespread presence of *p53* mutations in human cancer suggests that these markers may also be useful in the molecular detection of other tumor types.

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