Genetic Analyses of Non-Small Cell Lung Cancer in Jewish Israeli patients

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Abstract

Background: The contribution of the abnormal DNA mismatch repair system to non-small cell lung cancer tumorigenesis is controversial and has not been reported in Jewish Israeli patients. Similarly, the involvement of 3p deletions in NSCLC in the same population has not been assessed.

Objectives: To assess the contribution of the DNA-MMR system to NSCLC pathogenesis by analyzing microsatellite instability, and evaluate loss of heterozygosity at 3p rates in Israeli NSCLC patients.

Methods: Paired DNA from tumorous and non-tumorous tissue was extracted, and genotyping for MSI determination was carried out using the five Bethesda markers and for determining LOH two 3p markers were used. Genotyping was performed using polymerase chain reaction amplification and size separation on an ABI semiautomatic DNA sequencer, and the allelic patterns of tumorous and non-tumorous tissue were compared.

Results: Forty-four NSCLCs from 35 smokers and 9 non-smokers were analyzed, with 26 of the 44 (59%) at stage I disease. Using five microsatellite markers (D17S250, D5S346, D2S123, BAT-25, BAT-26) (known as Bethesda markers) for MSI determination, 6 of the 44 tumors (13.6%) exhibited MSI in at least one marker. Similarly, genotyping for LOH at chromosome 3p was performed using two markers (D3S4103, D3S1234) located at 3p14.2 l. With D3S4103, 33 of the 44 patients successfully analyzed were homozygous and therefore non-informative with respect to LOH. Using D3S1234, 33 of 36 patients (91.7%) were heterozygous, and 23 of these individuals' tumors (69.7%) displayed LOH. Unexpectedly, 4 of 33 tumors (12.1%) genotyped by D3S4103, and 16 of 36 tumors (44.5%) genotyped by D3S1234 showed a pattern of MSI, even though only one of these tumors showed a similar pattern when genotyped with the five consensus markers. Overall, 23 of 44 tumors (52.3%) demonstrated MSI on at least one marker, and 5 of these 23 tumors (21.7%) had MSI on two or more markers.

Conclusions: MSI using 3p markers and not the Bethesda markers occurs at a high rate and in early stages in Jewish NSCLC patients.

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Non-small cell lung cancer is a monoclonal tumor characterized by accumulation of multiple somatic genetic alterations [1]. One family of genes putatively involved in NSCLC tumorigenesis is the DNA mismatch repair genes. Inactivating mutations in these genes leads to accumulation of DNA replication errors hallmarked by genomic instability, as determined by comparing allelic pattern in tumorous tissue with the pattern in non-tumorous tissue using microsatellite markers [2]. In order to stratify and facilitate comparison of microsatellite instability rates reported by different laboratories using different microsatellite markers, a panel of five markers, coined the Bethesda markers, was chosen to assess and semi-quantify the existence of MSI [3]. The notion that defective DNA repair capacity is associated with NSCLC pathogenesis [4] is supported by reports of a high rate of MSI in histopathologically diverse lung cancer specimens [5–7]. In small cell lung cancer, 45–76% of primary tumors display MSI in the form of deletion or expansion of di- or tetranucleotide repeats [6,8]. In NSCLC, there are conflicting data regarding MSI rates (ranging from 0 to 69%) [5,6,9-11]. Another prevalent genetic abnormality in lung cancer is loss of genetic material (known as loss of heterozygosity) of chromosome 3p [12], reportedly detected in nearly 100% of SCLC [13] and in more than 50% of NSCLC [13].

While smoking clearly increases the risk for developing lung cancer, there are ethnic differences in the rate of smoking-related lung cancer risk: the risk being highest for African American and lowest, per cigarettes smoked, for Jewish individuals both in the United States and Israel [14,15]. Among African American males and females, the age-standardized rate of NSCLC is 115/100,000 and 57/100,000, respectively (http://www-dep.iarc.fr/globcan/globcan/html), whereas among the American Caucasian population it is 79.37/100,000 (males) and 52.28/100,000 (females). In Israel, the age-standardized rate for lung cancer in men and women in 1995 was 39.1/100,000 and 11.4/100,000, respectively [http://www-dep.iarc.fr/dataava/infodata.htm]. The reasons for these differences are not obviously apparent but may be attributed to genetic factors.

To gain insight into the molecular mechanisms of NSCLC in Jewish individuals and to evaluate the applicability of the Bethesda markers to determine MSI in these tumor types, we genotyped Jewish Israeli patients with NSCLC for MSI and 3p LOH using seven microsatellite markers.

NSCLC = non-small cell lung cancer
DNA-MMR = DNA mismatch repair
MSI = microsatellite instability
LOH = loss of heterozygosity

Patients and Methods

Sample collection and DNA extraction

Patients with a confirmed histopathologic diagnosis of NSCLC who were operated at the Sheba Medical Center from January 1999 to December 2001 were eligible for participation in the study. Tumor specimens were surgically resected and immediately stored at -70°C until DNA was extracted by using the QIAamp DNeasy tissue kit (Qiagen Ltd., Valencia, CA, USA) according to the manufacturer's recommended protocol. Peripheral blood DNA was extracted by using a DNA Isolation kit (PUREgene, Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. The study was approved by the institutional review board and each patient signed a written informed consent.

Table 1. Clinicopathologic and molecular data of 44 NSCLC patients

D3S1234	D3S4103	D5S346	D17S250	D2S123	BAT-26	BAT-25	Other cancer	Family history of cancer	Stage	Tape	Smoking Habits	Age (yrs)	Gender	Patient
LOH	+	–	–	–	–	–			T2N1M0	SQ	Y	60	F	1
LOH	MSI	–	–	–	–	–		Y	T4N0M0	BAC	N	78	F	3
MSI	MSI	–	–	–	–	–	Y		T1N0M0	SQ	Y	74	M	4
MSI	MSI	–	–	–	–	–			T1NOMO	AD	N	81	F	6
MSI	¥	–	–	–	–	–			T4N2M0	AD	N	56	F	7
LOH	¥	–	–	–	–	–	Y	Y	T2N2M0	AD	Y	66	F	10
+	MSI	–	–	–	–	–	Y	Y	T2N0M0	SQ	Y	64	F	11
MSI	¥	–	–	–	–	–			T4N0M1	AD	N	74	F	13
¥	¥	–	–	–	–	–			T2NOM0	SQ	Y	80	M	14
	¥	–	–	–	–	–		Y	T2N2M0	SQ	Y	74	M	16
¥	+	–	–	–	–	–	Y	Y	T3N0M0	AD	Y	78	F	17
¥	+	–	–	–	–	–		Y	T2N0M0	SQ	Y	79	M	18
LOH+MSI	+	–	LOH	–	–	–	Y		T1N0M0	AD	N	55	F	20
LOH	¥	–	–	–	–	–		Y	T3N0M0	AD	N	81	F	21
LOH	¥	–	–	–	–	–	Y	Y	T2N1M0	AD	Y	72	M	22
LOH	¥	LOH+MSI			–	–		Y	T2N0M0	LC	Y	60	M	24
LOH	¥	MSI	LOH	MSI	–	MSI	Y	Y	T2N1M0	SQ	Y	63	M	25
LOH	+	LOH	MSI	–	–	–			T2N0M0	BAC	N	69	M	26
MSI	+	–	–	–	–	–			T2N0M0	SQ	N	79	F	29
LOH	+	–	–	–	–	–			T2N0M0	AD	N	85	F	34
¥	¥	–	–	–	–	–			T2N0M0	AD	Y	76	M	37
–	+	–	LOH	–	–	–	Y		T2N1M0	SQ	Y	64	F	45
MSI	+	–	–	–	–	–			T1N0M0	SQ	Y	70	F	47
MSI	+	–	–	–	–	–			T1N0M0	SQ	Y	69	M	50
LOH+MSI	+	–	–	–	–	–			T2N0M0	SQ	Y	58	M	51
LOH+MSI	+	–	–	–	–	–			T3N0M0	SQ	Y	53	M	57
¥	+	–	–	–	–	–			T2N0M0	AD	Y	51	M	62
LOH	+	–	–	–	–	–			T1N0M0	SQ	Y	53	F	63
+	+	–	–	–	–	–			T2N0M0	AD	Y	73	M	73
LOH	+	–	–	–	–	–			T1NOM0	AD	Y	75	M	74
MSI	+	–	–	–	–	–	Y	Y	T1NOMO	SQ	Y	64	M	81
¥	+	–	–	LOH	–	–			T2NOMO	SQ	Y	59	M	85
MSI	+	–	–	–	–	–			T2NOMO	SQ	Y	69	M	87
+	+	–	–	–	–	–			T1NOMO	SQ	Y	79	M	89
LOH+MSI	+	–	–	–	–	–		Y	T2N2MO	SQ	Y	58	M	91
LOH	+	–	–	–	–	–			T3N1M0	SQ	Y	59	M	92
¥	+	–	–	–	–	–	Y	Y	T1N0M0	AD	Y	72	F	98
LOH	+	–	–	–	–	–			T2N1M0	AD	Y	77	M	101
MSI	+	–	–	–	–	–			T2N1M0	AD	Y	76	M	111
¥	+	–	–	–	–	–			T1NOMO	AD	Y	64	M	112
LOH	+	MSI	MSI	MSI	MSI	–			T2NOMO	SQ	Y	64	M	117
LOH	+	–	–	–	–	–			T2NOMO	SQ	Y	78	M	126
LOH+MSI	¥	–	MSI	–	–	–			T3N1MO	SQ	Y	67	M	127
LOH	+	–	MSI	MSI	–	–			T2N0M0	AD	Y	77	M	129

– = Normal

+ = Homozygous (not informative)

¥ = Unsuccessful analysis

MSI + LOH indicate that the changes occur together in the same area of the tissue

Polymerase chain reaction amplification

Tumor DNA and matching peripheral blood DNA were used to evaluate MSI status with five microsatellite markers: BAT-25, BAT-26, D2S123, D5S346, and D17S250 – the Bethesda marker panel. LOH of 3p genetic material was assessed using two microsatellite markers: D3S1234 and D3S4103, located within introns of the *FHIT* gene (3p14.2). Primer sequences are available through the Genome Database (http://www.gdb.org). PCR reactions were carried out using the PTC 60-100 M.J. Research thermal cycler (M.J. Research Inc., Watertown, MA) in a final volume of 10 µl, using 50–100 ng of genomic DNA as template, 1x PCR buffer (1.5 mM MgCl2), 200 nM each deoxynucleotide triphosphate, 10 pmol of each primer, and 1 unit of Taq DNA polymerase (Perkin Elmer Biosystems, Foster City, CA). Following a predenaturation step of 94°C for 5 minutes, 35 additional cycles were performed, with each cycle consisting of denaturation for 30 seconds at 94°C, 1 minute of annealing at 42–45°C, and 30 seconds of extension at 72°C, followed by a final extension step of 72°C for 5 minutes.

MSI and LOH analysis

PCR products were run in an ABI Prism 310 DNA Sequencer (PE Biosystems) according to the manufacturer's instructions. The data were collected automatically and analyzed by GeneScan 3.1 Software (PE Biosystems). When tumor DNA showed alleles that were not present in the corresponding non-tumorous tissue the tumor was classified as MSI-positive. If only one of the five Bethesda markers showed MSI, the tumor was classified as MSI-low (MSI-L), and if two or more markers showed MSI the tumor was classified as MSI-high (MSI-H). LOH was defined if allelic loss was noted in one allele in the tumor DNA when compared with the respective allele in the matched-paired leukocyte DNA.

Results

Patient and tumor characteristics

The study encompassed 44 tumors from 44 unrelated patients: 28 males and 16 females, 35 (79.5%) with a present or past history of smoking and 9 never-smokers, with the mean age at diagnosis of 68.9 ± 9 years (range 51–81 years). Histologic subtypes included 23 squamous cell carcinoma, 18 adenocarcinoma, 2 bronchioloalveolar carcinoma and one large cell carcinoma. Ten of 44 tumors (22.7%) were stage T1N0M0 and 16 (36%) stage T2NOMO. Thus the majority of tumors (26/44, 59%) were at stage I. Relevant clinical and pathologic information is shown in Table 1.

MSI analysis using the Bethesda markers

Six of 44 NSCLC tumors (13.6%) (# 24, 25, 26, 117, 127, 129; all males and 5 of the 6 were smokers) exhibited MSI in at least one of the five Bethesda markers [Figure 1, Table 1]. Three of these tumors (# 25, 117, 129) were defined as MSI-H (MSI in at least two markers), and three of these tumors (# 24, 26, 127) as MSI-L (MSI in only one marker). Three of the MSI-positive tumors were squamous cell carcinoma (# 25, 117, 127), and one each large cell carcinoma (# 24), bronchioloalveolar carcinoma (# 26), and adenocarcinoma (# 129). Four of the six MSI-positive tumors were staged at T2N0M0, one staged at T2N1M0 and one at T3N1M0.

Allelotyping with 3p14.2 markers

Using the marker D3S4103, 33 of the 44 patients who were successfully genotyped were homozygous in their non-tumorous DNA

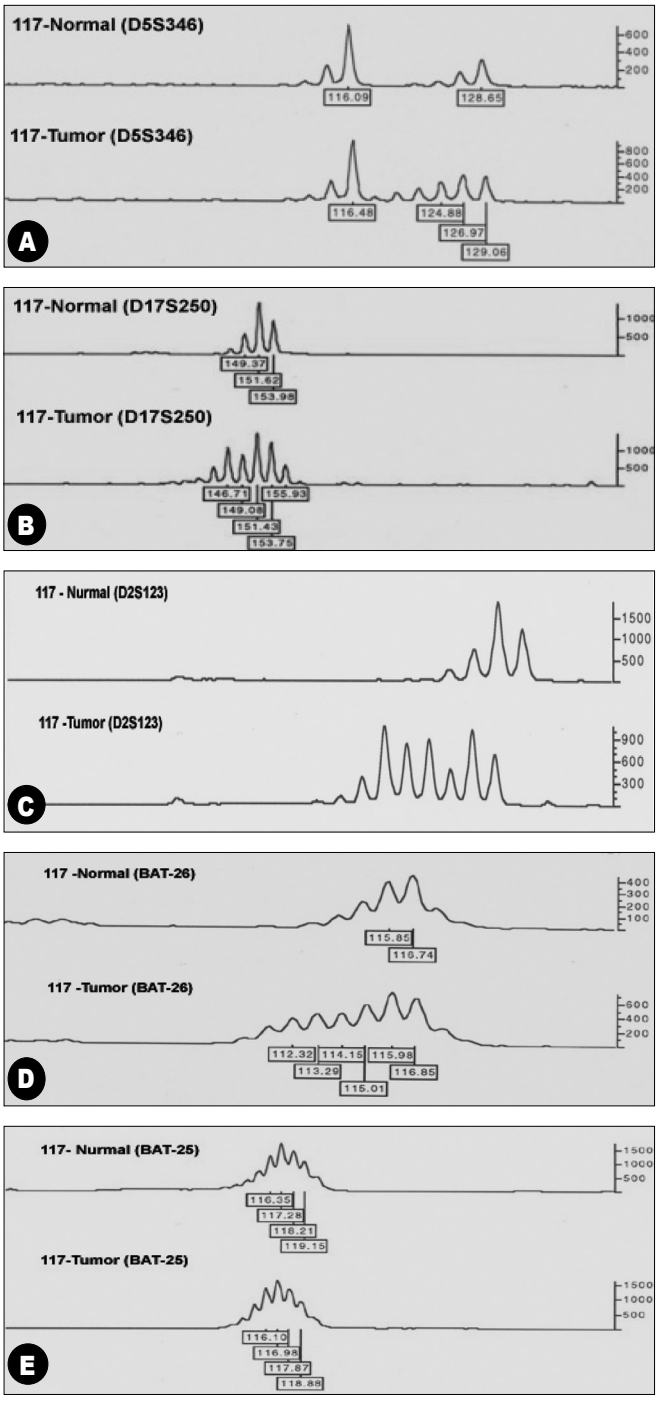

Figure 1. MSI analysis using five Bethesda markers. Patient # 117 shows instability at all markers **[A/B/C/D]** except BAT-26 **[E]**. For each marker, the bottom graph represents tumor DNA, and the top graph represents matching normal DNA.

PCR = polymerase chain reaction

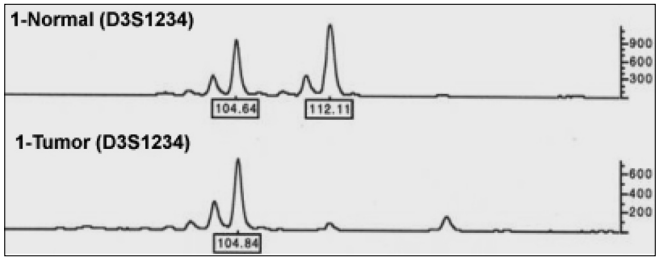

Figure 2. Patient # 1 shows LOH in the tumor DNA by using the marker D3S1234.

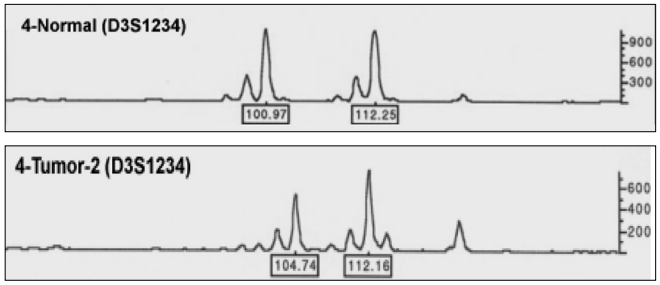

Figure 3. Patient # 4 shows pattern of MSI in the tumor DNA by using the marker D3S1234.

and therefore non-informative. Using the D3S1234 marker, 33 of 36 (91.7%) paired tumor constitutional DNA that underwent successful analysis were heterozygous (and therefore informative), and 23 of these individuals' tumors (69.7%) displayed LOH with that marker [Figure 2]. Unexpectedly, four tumors (12.1%) of a total of 33 examined by marker D3S4103, and 16 tumors (44.5%) of 36 examined by marker D3S1234 showed a pattern of MSI [Figure 3], even though only one of these tumors showed a similar pattern when genotyped with the five consensus markers (tumor # 117). Overall, 23 of the total 44 tumors examined (52.3%) demonstrated MSI in at least one marker and 5 (11.3%) showed an MSI pattern with two or more markers [Table 1]. In addition, 5 of 36 tumors (13.8%) showed a combined pattern of both LOH and MSI using D3S1234, and one tumor showed the same combined pattern using marker D5S346 [Figure 3].

Discussion

In the present study, using the Bethesda markers, only 6 of 44 NSCLC tumors from Israeli patients (13.6%) displayed MSI, whereas using 3p markers more than 50% of the tumors displayed MSI with at least one marker. In addition, use of mononucleotide repeats (e.g., BAT 25, BAT 26) failed to adequately detect MSI in NSCLC in the present series. The reported rate of MSI in NSCLC in ethnically diverse populations ranges widely from 0% to more than 69% [5,6,10,11,14,16]. One of the factors that may account for this wide range of reported MSI in this neoplasm is the number and chromosomal locations of the specific markers used: the highest rates of MSI were reported from within regions containing genes known or putatively involved in lung cancer pathogenesis – 3p14 (within the *FHIT* gene) [17] and 9p21 (within the *P16* gene) at 10q24 (a region showing a high rate of allelic imbalance in many cancer types) [18]. Thus, unlike MSI in the context of hereditary non-polyposis colon cancer, which is generalized, MSI in NSCLC is more limited and may be site-specific. Secondly, the type of repeat sequences varies widely: mono-, di-, tri- and tetranucleotide repeats were used in various studies. Consistent with the data in the present study, most studies reported that the use of tetranucleotide repeats yields higher rates of MSI than the use of mono- or dinucleioptide repeats [19]. Furthermore, Ahrendt and co-workers [20] compared the rate of MSI as determined by the five Bethesda consensus markers with that scored by use of 13 tetranuclotide repeats from different chromosomal loci in 88 NSCLC samples. While the use of the tetranucleotide repeats yielded an MSI rate of 35% (31/88), only 1 of the 31 MSI-positive tumors showed a pattern of MSI-L with the Bethesda markers.

Taken together, it seems logical to recommend using tetranucleotide markers or markers from within the *FHIT* (3p14.2) or *P16* genes (9p21) to detect and score MSI in NSCLC.

In the preset study there was no correlation between the MSI genotype and smoking status, age at diagnosis, histopathologic subtype, or family history of cancer [Table 1]. Previous studies correlating MSI in NSCLC with clinical and pathologic parameters report either no association with any of the above-mentioned ones, or a higher rate of MSI in adenocarcinomas [18], and in females with a family history of cancer, mostly gastric and lung [21]. Despite the lack of correlation with most clinical parameters, two of the six patients in the present study with a MSI-positive pattern using the Bethesda markers died of lung cancer within one year of the early-stage diagnosis, suggesting that MSI as determined by these specific markers may be associated with a worse prognosis, or a more aggressive clinical course than tumors not displaying MSI. Replication error-positive genotype using 2p and 3p markers was reportedly associated with a significantly worse 5 year prognosis in stage I NSCLC [5,22]. Similarly, a MSI-positive genotype with 10q24 markers had a significantly shorter disease-specific survival [18].

Conclusions

Using the Bethesda markers to determine MSI in NSCLC leads to underestimation of the true rate, and there seems to be a need to define a set of markers (preferably of the tetranucleotide variety) that can be used for stratification and standardization of the reported rates in ethnically diverse populations. In addition, MSI genotype may be associated with a worse prognosis in stage I NSCLC, but certainly more prospective studies with standardized markers are needed.

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