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Ras oncogene point mutations in bladder cancer resistant to cisplatin

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Summary. Bladder tumors respond to cisplatin-based chemotherapy in 68% of cases, but only 30% will have a durable response. Recent studies have suggested that *ras* point mutations may produce cisplatin drug resistance in cell lines. To determine the role of *ras* point mutations in human tumors resistant to cisplatin, we evaluated ten tumors exposed to cisplatin and eight untreated bladder tumors for *ras* point mutations. Using polymerase chain reaction DNA amplification and allele-specific oligo-hybridization, we found that only one of the ten treated tumors and none of the untreated tumors harbored a ras point mutation. We conclude that *ras* point mutations occur infrequently in untreated bladder tumors and do not appear to correlate with cisplatin drug resistance in vivo.

Key words: *Ras* point mutations – Cisplatin-resistant bladder cancer

Cisplatin is the most active agent in chemotherapeutic regimens for bladder cancer. A 40–70% overall and a 15–36% complete response rate is reported with a variety of cisplatin based chemotherapeutic programs [10, 17, 22]. Primary bladder tumors and metastatic lesions have similar response rates [17]. Unfortunately, 68% of complete responders ultimately relapse [17]. This implies that some bladder tumors acquire a drug-resistant phenotype in response to therapy, possibly via alterations in drug-sensitive cells or by the selection of intrinsically drug-resistant cells that existed prior to treatment and proliferated as drug-sensitive cells died. Several mechanisms for cisplatin drug resistance have been found in vitro, including glutathione or metallothionein overexpression and *ras* oncogene point mutations [5, 7, 16].

Point mutations in codons 12, 13, or 61 of the *ras* proto-oncogene can cause NIH 3T3 cells to express a malignant phenotype [15]. The clinical importance of *ras* oncogenes in human tumors is unknown. They may play a role in tumorigenesis in several human cancers, including colon, pancreas and germ cell tumors [2]. Recently, *ras* point mutations have been implicated in causing cellular resistance to cisplatin, in vitro. The EJ bladder cancer cell line, which has a point mutation in the 12th codon of H-*ras*, is highly resistant to cisplatin compared with non-mutation-containing cells. This phenomenon has been reproduced in NIH 3T3 cells transfected with mutated *ras* genes. These transfected cells are over 100-fold more resistant to cisplatin compared with NIH 3T3 cells transfected with normal human DNA [16].

Using NIH 3T3 transfection assays, previous studies have found that 7–17% of untreated bladder tumors harbor a *ras* point mutation [3, 20]. To investigate whether *ras* point mutations occur more commonly in bladder tumors treated with cisplatin and therefore possibly play a role in drug resistance in vivo, we evaluated treated and untreated bladder tumors for the presence of *ras* point mutations using the more sensitive polymerase chain reaction (PCR) DNA amplification and allele-specific oligo-hybridization technique.

Materials and methods

Tumor procurement

Bladder tumor tissue was obtained from 18 patients undergoing radical cystectomy at Memorial Sloan-Kettering Cancer Center. All bladder tumors were transitional cell carcinomas. The specimens were snap-frozen in liquid nitrogen and then stored at −70°C. Ten of these tumors were exposed to at least one dose of cisplatin chemotherapy, usually administered in combination with methotrexate, vinblastine and Adriamycin, and are referred to as "treated." The other eight bladder tumors were never exposed to chemotherapy and are referred to as "untreated."

Polymerase chain reaction

High-molecular-weight DNA was isolated from fresh tumor tissues following methods already described elsewhere and dissolved in double-distilled water [8]. The DNA was subjected to PCR amplification as previously described, with minor modifications [13]. The reaction mixture contained 1 μg of genomic DNA and 660 ng of each

Table 1. Amplimers

Amplimer[a]	Sequence[b]	Target	Fragment size (bp)
HA-12	GACGGAATATAAGCTGGTGG	Ha-*ras*12	63
HB-12	TGGATGGTCAGCGCACTCTT		
HA-61	AGACGTGCCTGTTGGACATC	Ha-*ras*61	73
HB-61	CGCATGTACTGGTCCCGCAT		
KA-12	GACTGAATATAAACTTGTGG	Ki-*ras*12	108
KB-12	CTATTGTTGGATCATATTCC		
KA-61	TTCCTACAGGAAGCAAGTAG	Ki-*ras*61	128
KB-61	CACAAAGAAAGCCCTCCCCA		
NA-12	GACTGAGTACAAACTGGTGG	N-*ras*12	109
NB-12	CTCTATGGTGGGATCATATT		
NA-61	GGTGAAACCTGTTTGTTGGA	N-*ras*61	103
NB-61	ATACACAGAGGAAGCCTTCG		

[a]Amplimers: synthetic oligodeoxynucleotides used to amplify DNA segments in vitro. Letter "A" refers to amplimers 5′ of the target sequence, and letter "B" to amplimers 3′ of the target sequence
[b]All the amplimers are 20 nt long
[c]Fragment length includes the amplimer sequences

of the appropriate oligonucleotide amplimers flanking the 5′ and 3′ regions of the mutation site in question (Table 1): 3.5 m*M* MgCl, 50 m*M* KCl, 10 m*M* Tris-Cl pH 8.3, 0.1% (w/v) gelatin, 200 μ*M* of each dNTP and 2.5 units of *Taq* polymerase (Perkin-Elmer-Cetus, Norwalk, Conn., USA). The amplimers for the 12th codon of each *ras* gene were also used to amplify the 13th codon. The reaction mixture was mixed and 50 μl of mineral layered on top, after which the tube was placed in the thermocycler. The PCR amplification was performed with a Perkin-Elmer-Cetus DNA thermal cycler in the following sequence: 94°C for 1 min to denature the DNA, 37°C for 5 min to allow the amplimers to anneal, 72°C for 5 min for DNA polymerization. This cycle was repeated 30 times. To check for adequate amplification of the specific segment, 10 μl of each reaction mixture was electrophoresed on a 4% NuSieve agarose gel in 1× TBE buffer (89 m*M* Tris borate, 89 m*M* boric acid, 2 m*M* EDTA) at 70 V for 4 h. The gel was then stained in ethidium bromide (5 μg/ml) for visualization of the approriately sized segment under UV illumination.

Oligo-Hybridization

For oligo-hybridization, 10 μl of the PCR product was denatured in 0.25 *N* NaOH for 10 min and then slot-blotted (Hybri-slot manifold) onto nylon filters (Sure blot) under vacuum. The DNA was immobilized on the filter by baking in a vacuum oven at 80°C for 1 h and then prehybridized for 30 min at 56°C in 3 *M* tetramethylammonium chloride/50 m*M* Tris pH 8/2 m*M* EDTA pH 8/0.3% NaDodSO$_4$/100 μg/ml denatured salmon sperm and 5× Denhardt's solution = 0.02% Ficoll/0.02% polyvinylpyrolidone/0.02% bovine serum albumin). Oligonucleotide probes specific for each possible point mutation for the 12th, 13th and 61st codons of the H-, N- and K-*ras* genes were made using the Applied Biosystems oligonucleotide synthesizer (Table 2) [19]. Then, 10 ng of germline or mutation-specific oligonucleotide probe was end-labeled with p^{32}ATP by T$_4$ Kinase to at least 10^8 cpm/μg probe, and 1×10^6 cpm of probe was added to the prehybridization mixture and incubated at 56°C for 1 h. Filters were then washed twice at room temperature for 10 min in 2× SSPE (1× SSPE = 10 m*M* sodium phosphate pH 7.2/0.18 *M* EDTA pH 8/0.1% SDS). Subsequently, the filters were washed in the hybridization buffer minus the Denhardt's solution and salmon sperm and then incubated in this solution for 45 min at 60°C. The filters were then washed twice at room temperature for 10 min in 2× SSPE. Finally the filters were exposed to Kodak XR film at −70°C for 4–24 h.

Table 2. Complete list of the oligomers used to analyze *ras* gene mutations

Oligomer[a]	Sequence[b]	
H12	GTGGGCGCCggcGGTGTGGG	
H12-p1	---------ngc--------	n=A, C, T
H12-p2	---------gnc--------	n=A, C, T
H12-p3	---------ggn--------	n=A, G, T
H13	GTGGGCGCCGGCggtGTGGG	
H13-p1	------------ngt-----	n=A, C, T
H13-p2	------------gnt-----	n=A, C, T
H13-p3	------------ggn-----	n=A, C, G
H61	TACTCCTCctgGCCGGCGGT	
H61-p1	--------ntg---------	n=A, G, T
H61-p2	--------cng---------	n=A, C, G
H61-p3	--------ctn---------	n=A. C, T
K12	CCTACGCCaccAGCTCCAAC	
K12-p1	--------ncc---------	n=C, G, T
K12-p2	--------anc---------	n=A, G, T
K12-p3	--------acn---------	n=A, G, T
K13	CCTACgccACCAGCTCCAAC	
K13-p1	-----ncc------------	n=A, C, T
K13-p2	-----gnc------------	n=A, G, T
K13-p3	-----gcn------------	n=A, G, T
K61	TACTCCTCttgACCTGCTGT	
K61-p1	--------ntg---------	n=A, C, G
K61-p2	--------tng---------	n=A, C, G
K61-p3	--------ttn---------	n=A, C, T
N12	GGAGCAggtGGTGTTGGGAA	
N12-p1	------ngt-----------	n=A, C, T
N12-p2	------gnt-----------	n=A, C, T
N12-p3	------ggn-----------	n=A, C, G
N13	GGAGCAGGTggtGTTGGGAA	
N13-p1	---------ngt--------	n=A, C, T
N13-p2	---------gnt--------	n=A, C, T
N13-p3	---------cgn--------	n=A, C, G
N61	TACTCTTCttgTCCAGCTGT	
N61-p1	--------ntg---------	n=A, C, G
N61-p2	--------tng---------	n=A, C, G
N61-p3	--------ttn---------	n=A, C, T

[a]Refers to oligodeoxynucleotides spanning the specified codon the *ras* gene; p1, p2, and p3 indicate groups of mutation-specific oligomers, complementary to sequences mutated at the first, second, or third nucleotide of the codons
[b]Sequence of the oligomers

Results

None of the eight untreated tumors was found to have a mutation in the *ras* oncogene (data not shown). Of the ten treated tumors, only one harbored a *ras* oncogene point mutation (Table 3). This tumor had a mutation in the second base pair of codon 61 in the H-*ras* gene (Fig. 1). We found no evidence of mutations in the N-*ras* or K-*ras* genes. From dilutional assays in which DNA from the T24 cell line, which has a *ras* point mutation at the H12 codon, was mixed with placental DNA, we identified the *ras* point mutation if at least 5% of the total DNA sample contained T24 DNA (data not shown). Thus, it is unlikely that dilution of tumor cells with normal cellular elements could account for the low prevalence of *ras* point mutations in our treated bladder tumors. The patient with a *ras* point mutation had no response to one cycle of cisplatin,

Table 3. Treated tumors screened for *ras* point mutations

Specimen number	Treatment[a] (no. of courses)	Response[b]	*Ras*[c] mutation
T1	MVAC (4)	PR	–
T2	MVAC (4)	PR	–
T3	MVAC (1)	NR	–
T4	MVAC (2)	CR relapse	–
T5	MVAC (4)	PR	–
T6	MVAC (4)	NR	–
T7	MVAC (6)	NR	–
T8	CMV (1)	NR	+(H61)
T9	MVAC (3)	NR	–
T10	MVAC (4)	CR relapse	–

[a]MVAC, Methotrexate, vinblastine, Adriamycin, and cisplatin; CMV, cisplatin, methotrexate, and vinblastine
[b]CR, Complete response; PR, partial response; NR, no response
[c]–, No mutations in H-, K- or N-*ras* genes; +, mutation present in specified codon

methotrexate and vinblastine combination chemotherapy. The other four nonresponsive tumors had germline *ras* configurations. Therefore, it appears that *ras* point mutations occur infrequently in tumors exposed to cisplatin.

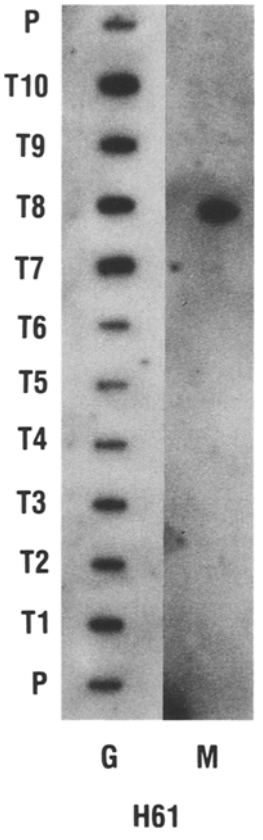

Fig. 1. Oligospecific hybridization with probes for mutations in the second base pair of the H61 codon. Tumor T8 contains a mutation at this site. *P*, Placenta DNA; *G*, Germline; *M*, mutation

Discussion

Drug resistance is a major problem in oncology. Several chemotherapeutic agents produce significant tumor reduction in a variety of cancers, but in most instances the tumor regrows and eventually results in the demise of the patient. Cisplatin, an electrophilic toxin, is one of the most effective agents in solid tumors. Cisplatin acts by causing intrastrand DNA crosslinking, thereby disrupting RNA and DNA synthesis [23]. Drug resistance to cisplatin occurs via two mechanisms: improved DNA repair and decreased drug accumulation in the nucleus.

Cell lines resistant to cisplatin have an increased capacity to repair DNA damage caused by cisplatin [9]. There is evidence that glutathione, which is overexpressed in cells that are resistant to cisplatin, may play a role in DNA repair processes following radiation [21]. Additionally, glutathione-depleting agents can increase the sensitivity of cells to cisplatin [5]. Sequestration of cisplatin in the cytoplasm will decrease the intranuclear concentration of the drug and prevent the formation of DNA adducts. Metallothionein, which is the most prominent intracellular protein sulfhydryl, binds to heavy metals and electrophilic compounds, including cisplatin, in the cytoplasm [1]. Several studies have revealed increased metallothionein protein in cell lines resistant to cisplatin [1, 7]. It is unclear, however, how activated *ras* genes produce cisplatin drug resistance. Several investigators have observed that cells transfected with a mutated *ras* gene are resistant to the effects of cisplatin in vitro. These transfected cell lines overexpress either glutathione or metallothionein [6, 14, 18]. The mechanism by which mutated *ras* genes produce overexpression of these proteins is unknown.

Tumors that are initially sensitive to cisplatin may undergo genetic changes to produce a drug-resistant phenotype, or a small population of intrinsically resistant cells may be present and proliferate as the drug-sensitive cells die. From previous studies using the NIH 3T3 assay to detect *ras* point mutations approximately 10% of untreated bladder tumors had a mutation [3, 20]. We utilized the more sensitive PCR technique to ensure identification of *ras* point mutations even if only a small population of cells had the mutation. In the eight untreated tumors we did not find any *ras* point mutations, which considering the sample size means a similar incidence to those in previous reports, despite using a more sensitive assay. Our finding that only one of ten treated bladder tumors has a *ras* point mutation suggests that other mechanisms of cisplatin drug resistance are involved in bladder cancer.

There are few data to suggest that *ras* genes play a role in cisplatin drug resistance in other human tumors. Germ cell tumors are very sensitive to cisplatin whether it is given as a single agent or in combination with other drugs [11]. In one study a 65% incidence of *ras* point mutations in germ cell tumors was found [4]. Specifically, seminomas, which are exquisitely sensitive to radiation and cisplatin, have a 59% incidence of *ras* point mutations.

Futhermore, renal cell carcinoma has a very low incidence of *ras* point mutations, yet is highly resistant to cisplatin [12].

Because the mutated H-*ras* containing bladder cancer cell line, EJ, is resistant to cisplatin, we thought this may be an important mechanism of cisplatin drug resistance in bladder cancer, in vivo. However, with the multiple mechanisms for cisplatin resistance, it is possible that *ras* point mutations are not commonly involved in provided cisplatin drug resistance in vivo. One of our tumors did have a *ras* point mutation that could have contributed to the cisplatin-resistant phenotype in this tumor. Additonal tissue to determine whether there was overexpression of glutathione of metallothionein in this tumor was not available. However, our observation that nine out of ten tumors exposed to cisplatin did not have a *ras* mutation, coupled with the findings in germ cell tumors and renal cell carcinoma, suggest that *ras* point mutations do not play a significant role in cisplatin drug resistance in human tumors in vivo.

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