NEOPLASIA

Mutation of the p53 Gene Is Not a Typical Feature of Hodgkin and Reed-Sternberg Cells in Hodgkin's Disease

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Point mutations of the p53 tumor suppressor gene are a frequent finding in human carcinomas and are thought to be an important oncogenic event. In non-Hodgkin lymphomas, p53 mutations occur in a minor fraction of cases. However, conclusive data are still lacking for Hodgkin's disease (HD) where the analysis meets technical problems. The neoplastic tumor cell clone in HD is represented by the large Hodgkin and Reed-Sternberg (HRS) cells, which account for only a minority of all cells in the tumor tissue (often <1%). To identify putative HRS cell-specific mutations, single HRS cells were micromanipulated from frozen tissue sections of HD biopsy specimens. Exons 4 to 8 of the p53 gene (in which more than 90% of p53 mutations associated with human neoplasms occur) were amplified from these single cells and sequenced. Mutations of p53 were not found in HRS cells of any of 8 cases of HD analyzed. We conclude that mutation of the p53 gene is only rarely, if at all, involved in the pathogenesis of HD.
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HODGKIN AND REED/STERNBERG (HRS) cells represent the malignant tumor cells of Hodgkin's disease (HD). In contrast to most other neoplasms, the tumor clone in HD accounts for only a minor fraction of all cells in the tumor tissue.[1] It has recently been shown that the HRS cell population is derived from mature B cells.[2] The presence and pattern of somatic mutations in rearranged immunoglobulin variable region genes amplified from HRS cells suggested that the HRS cell precursor is a germinal center (GC) B cell which has lost the capacity to express antigen receptor. GC B cells with "crippled" antigen receptor die by apoptosis under normal circumstances. The HRS cell precursor might initiate the apoptotic pathway, but survive because of an unknown transforming event.[2,3]

Loss of function of the p53 protein seems to be important in the oncogenesis of human cancer because inactivating point mutations of the p53 gene are found in a high proportion of colorectal, lung, and breast cancers.[4] In carcinoma cells, mutation of one allele is usually accompanied by deletion of the second allele.[5,6] Mutations of the p53 tumor suppressor gene have also been investigated in lymphomas and were found in a minority of non-Hodgkin lymphomas (NHL) on primary disease manifestation, but with higher frequency in relapsed cases.[7-9]

Whether alteration of the p53 gene plays a role in malignant transformation of HRS cells in HD is a long debated question. The search for mutations in the DNA of HRS cells is hampered by the scarcity of these cells in the tumor tissue (often <1% of all cells). Mutations have been detected in a minor fraction of HD cases by the single-strand conformation polymorphism (SSCP) technique in DNA extracted from whole-tissue specimens.[10-16] However, it was not possible to unequivocally show that the mutated sequences indeed originated from the HRS cell population. By amplification of p53 cDNA from single HRS cells isolated from cell suspensions of fresh tumor tissue in 3 cases of HD, Trümper et al showed that (part of) the HRS cell clone of 3 patients carried a mutated p53 allele.[17,18]

To investigate whether p53 mutations frequently occur in HD, we micromanipulated single HRS cells from frozen tissue sections of 8 patients suffering from classical HD. Exons 4 to 8 of the p53 gene, where more than 90% of mutations of p53 are found,[4,7] were amplified from the genomic DNA of these single cells in a multiplex polymerase chain reaction (PCR) and sequenced.

MATERIALS AND METHODS

Tissue samples and cell line CO. Patient data are summarized in Table 1. In all cases of primary HD, enlarged cervical lymph nodes were analyzed except for case 8, which was an axillary lymph node. These cases were selected to represent the typical histology of nodular sclerosing (NS) or mixed cellularity (MC) HD. The material of the 2 relapsed cases, 6 and 7, was a paraaortal and an inguinal lymph node biopsy, respectively. In all 8 cases, the HRS cells were positive for CD30 and except for 1 case (relapsed case 6), the HRS cells also expressed CD15. (No material for CD15 staining was available for case 4 [Table 1].) Cell line CO[10,19] was obtained from V. Diehl (University of Cologne, Cologne, Germany).

Sorting of peripheral blood cells. Peripheral blood mononuclear cells (PBMC) were isolated from blood of a healthy Caucasian donor by centrifugation over Ficoll Paque R (Pharmacia, Freiburg, Germany) and stained with anti–$\alpha\beta$-TCR-PE (Immunotech, Hamburg, Germany) and anti–CD3-FITC (Becton Dickinson, Heidelberg, Germany). Single $\alpha\beta$-TCR$^+$/CD3$^+$ cells were deflected into PCR tubes using a FACS 440 (Becton Dickinson) as described.[20]

Immunostaining of frozen tissue sections and cytospins and micromanipulation. Eight-micrometer–thick frozen sections of tumor tissue were mounted onto glass slides, air-dried, and fixed in acetone for 10 minutes. PBMC were mixed with CO cells, centrifuged onto glass slides, air-dried, and fixed in acetone for 10 minutes. Sections and cytospins were stained with antibodies against p53 (CM1; NovoCastra, Newcastle-Upon-Tyne, UK), CD15 (LeuM1; Becton Dickinson), CD20 (L26; Dako, Glostrup, Denmark) or CD30 (BerH2; Dako) as described previously.[20] Sections were overlaid with Tris-buffered saline containing 5 mg/mL collagenase H (Boehringer Mannheim, Mannheim, Germany). Single cells were mobilized from the sections with the help of hydraulic micromanipulators (Narishige, Japan) as described.[21]

Table 1. Patient Data

Case	HD Manifestation	HD Subtype	Age	Sex	HRS Cells		
					CD30	CD15	p53†
1	Primary	NS	51	Female	+	+	+
2	Primary	NS	27	Female	+	+	+
3	Primary	MC	36	Male	+	+	ND
4	Primary	NS	17	Female	+	ND	+
5	Primary	MC	69	Male	+	+	+
6	First relapse	?*	40	Male	+	−	+
7	First relapse	MC	17	Male	+	+	ND
8	Primary	NS	18	Female	+	+	+

Abbreviations: HD, Hodgkin's disease; ND, not done; NS, nodular sclerosing; MC, mixed cellularity.

*Classical HD, subtype of primary manifestation unknown.

†"+" indicates that at least 80% of HRS cells are positive for p53 protein.

Amplification of exons 4 to 8 of the p53 gene from single cells.
Single cells in 20 μL Expand HF PCR buffer (Boehringer Mannheim) were incubated with 0.25 mg/mL proteinase K (GIBCO Life Technologies, Eggenstein, Germany) for 2 hours at 50°C. The enzyme was inactivated by heating to 95°C for 10 minutes. The first round of amplification was performed in the same reaction tube in a 50 μL volume containing 1X Expand HF buffer; 2 mmol/L $MgCl_2$, 125 μmol/L each of dATP, dCTP, dTTP, and dGTP; 16.6 nmol/L each first round primer (4UA, 4D, 56UA, 56D, 78UA, and 78D; Table 2); and 2.5 U of Expand HF polymerase mix (Boehringer Mannheim). Thermal cycling was performed in a Biometra Unoblock (Biometra, Göttingen, Germany). An initial cycle of 95°C for 2 minutes, a pause at 80°C during which the enzyme was added, 61°C for 30 seconds, and 72°C for 50 seconds was followed by 35 cycles of 95°C for 60 seconds, 61°C for 30 seconds, and 72°C for 50 seconds, followed by 72°C for 5 minutes. For the second round of amplification, 1 μL of the first-round reaction mixture was added to 3 separate reaction mixes each containing 1 primer pair (4UB and 4D, 5/6UB and 5/6D, 7/8UB and 7/8D; Table 2). Second-round reactions were performed in a volume of 50 μL with 20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 125 μmol/L each of dATP, dCTP, dTTP, and dGTP, 125 nmol/L each primer and 1.25 U Taq DNA polymerase (GIBCO) in 96-well plates (Costar, Bodenheim, Germany) using a Biometra Uno-Thermoblock. All components were added on ice and the 96-well plate was placed into the thermocycler after the block had reached denaturing temperature (95°C). The cycle program consisted of 1 cycle of 95°C for 3 minutes, 61°C for 30 seconds, and 72°C for 90 seconds, followed by 44 cycles of 95°C for 60 seconds, 61°C for 30 seconds, and 72°C for 60 seconds, followed by a 5-minute incubation at 72°C.

Sequence analysis. PCR products were directly sequenced from both sides using the second-round primers. Cycle sequencing was performed using the Ready Reaction Dye Deoxy Terminator cycle sequencing kit (Perkin Elmer, Foster City, CA) and an ABI 377 automatic sequencer. DNASIS software (Pharmacia) and the GenBank data library (release 93.0) were used to analyze the sequences.

Table 2. Primers for Amplification of Exons 4 to 8 of the p53 Gene From Single Cells

4UA	5′-ACT-GAA-GAC-CCA-GGT-CCA-GAT-GAA-3′
4UB	5′-TTC-TGT-CCC-TTC-CCA-GAA-AAC-CTA-3′
4D	5′-AGG-GTG-AAG-AGG-AAT-CCC-AAA-GTT-3′
5/6UA	5′-TGT-TCA-CTT-GTG-CCC-TGA-CTT-TCA-3′
5/6UB	5′-CTC-TGT-CTC-CTT-CCT-CTT-CCT-ACA-3′
5/6D	5′-AGG-GCC-ACT-GAC-AAC-CAC-CCT-TA-3′
7/8UA	5′-CAA-GGC-GCA-CTG-GCC-TCA-TCT-T-3′
7/8UB	5′-CCT-CAT-CTT-GGG-CCT-GTG-TTA-TCT-3′
7/8D	5′-ACC-GCT-TCT-TGT-CCT-GCT-TGC-TTA-3′

RESULTS

Sections of paraffin-embedded tissue specimens were immunostained for p53 protein in 6 of the 8 cases investigated. In all 6 cases, at least 80% of the HRS cells were positive for p53 protein (Table 1).

Because more than 90% of all point mutations found in the p53 gene of malignant cells are located within the downstream part of exon 4 and in exons 5 to 8,[4,7] sequence analysis was focused onto these exons. Amplification of exon sequences from single cells was performed by seminested PCR. The primers hybridized in the intronic sequences flanking the exons. Three segments of the p53 gene were amplified with different primer pairs, the first (E4) containing the downstream part of exon 4, the second (E5/6) containing exons 5 and 6, and the third (E7/8) containing exons 7 and 8. In the first round of amplification, all 6 primers were used together in the same tube, while second round amplifications were performed in separate reactions. As a positive control for efficient amplification from single genomes, single cells (peripheral blood T cells from a healthy donor) were sorted into PCR tubes by flow cytometry and analyzed by PCR. All 3 segments of the p53 gene could be amplified from 81 of 87 tubes (90%). The remaining 6 tubes were negative for all 3 products, which is most likely because of failure of the single-cell deflection.

Potential alterations of the p53 gene in HD may occur as point mutations on 1 allele with concomittant loss of the second allele. This is typically found in carcinomas. Alternatively, the 2 alleles may be affected by 2 different point mutations. In the latter situation, mutations are more difficult to detect by amplification and sequencing because mixed sequences result if both alleles are amplified from a given cell. To validate that the method used in the present study can detect a point mutation in micromanipulated single cells, which also harbor an unmutated allele of the p53 gene, a control experiment was performed using cell line CO. CO cells harbor 2 mutated alleles[10] with 1 point mutation located in exon 5 and a second in exon 8 (codons 175 and 282, positions 13203 and 14513 of the published wild-type sequence of the p53 gene,[22] accession no. X54156). Cells of the CO line are large and express CD30 but not CD20. CO cells were mixed with PBMC from a healthy donor in a ratio of 1:100 and the mixture was centrifuged onto glass slides such that the CO cells were tightly surrounded by PBMC. Cytospins were stained for CD30 or CD20. Large CD30+ cells were

micromanipulated from CD30-stained cytospins and analyzed by single-cell PCR. Small CD20+ cells manipulated from CD20-stained cytospins served as controls. Exon pairs 5/6 and 7/8 could be amplified from 7 of 10 CD30+ cells. For the majority of the CD30+ cells, the sequencing chromatograms showed a double band at positions 13203 and 14513, indicating coamplification of both the mutated and the unmutated sequence (Table 3). The double band was detectable unambiguously in forward and reverse sequencing reactions. In rare instances, only the mutated sequence was obtained (Table 3). The CD20+ control cells yielded unmutated sequences only. These results show that the combination of micromanipulation and single-cell PCR reliably detects point mutations in single cells even if the cells are heterozygous for this mutation.

Tissue samples from 8 cases of HD were analyzed, 6 of which were biopsy specimens taken at primary manifestation of the disease. Two biopsy specimens were taken at first relapse (Table 1). HRS cells were identified in frozen sections of tumor tissue as large, multinucleated CD30+ cells. Ten to 12 HRS cells were micromanipulated for each case and subjected to single-cell amplification. To be able to identify HRS cell-specific mutations, non-HRS cells (CD20+ B cells) were micromanipulated from adjacent sections and analyzed in parallel as controls. Aliquots of the buffer covering the section during the micromanipulation procedure served as negative controls in the PCR. These controls were always negative except for 1 aliquot in case 4 from which all 3 p53 gene segments were amplified. This was probably due to cellular contamination during the micromanipulation procedure because 14 additional buffer aliquots from the same case were negative. Each of the 3 p53 gene segments (E4, E5/6, E7/8) was amplified from at least 4 different HRS cells (with the exception of segment E7/8 in case 8; Table 4). In most cases, each gene segment was amplified from 6 to 8 different HRS cells. PCR products were directly sequenced from both sides.

Table 3. Validation Experiment: Detection of p53 Point Mutations in Single Micromanipulated CD30+ CO Cells

Cell	Exon 5 (codon 175) Position 13203	Exon 8 (codon 282) Position 14513
*CD30+		
1	A/G	T/C
2	A/G	T/C
4	A/G	T/C
7	A/G	T/C
8	A	T/C
9	A/G	T/C
10	A	T
†CD20+		
36	G	C
38	G	
39	G	C
40	G	C
41	G	C
Wild-type p53‡	G	C
Cell line CO	A	T

*CO cells.
†B cells isolated as controls.
‡Accession no. X54156.

No sequence differences were detected between HRS and B cells in the downstream part of exon 4, over the complete length of the coding regions of exons 5 to 8 and the exon-intron boundaries in any of the 6 primary nor the 2 relapsed cases analyzed. All exonic sequences amplified from the HRS and B cells were identical to the published sequence of the p53 gene.[22] The chromatograms were also analyzed for double bands indicating heterozygosity at a particular position; however, no mixed sequences could be identified.

In 4 of the 8 cases (case 1, 2, 3, and 8), sections of the same tissue samples had previously been used for an analysis of the HRS cell population for the presence of immunoglobin (Ig) gene rearrangements (case 2, 5, 7, and 4 in Kanzler et al,[2] respectively) by single-cell PCR. In all these cases, the CD30+ HRS cells identified using the same criteria as applied in the present study and micromanipulated under the same conditions, were shown to harbor clonal Ig gene rearrangements.[2] In the present study, the V_H3 gene rearrangement of the HRS cell clone of case 8 was coamplified along with the p53 exons to provide direct evidence that the p53 PCR products originated from the clonal HRS cell population of this case. This was done by addition of a V_H3-specific and a J_H-specific primer (primers V_H3 and $3'J_H6$)[2] to the reaction mix in the first round of amplification. The clonal V_H3 rearrangement was obtained from 6 of 16 HRS cells (Table 5). Five of 8 HRS cells from which unmutated p53 PCR products were obtained were also positive for the clonal V_H3 gene rearrangement. One cell was excluded from the sequence analysis because a clonally unrelated V_H3 gene rearrangement was coamplified with the p53 exons. This cell likely represents cellular contamination. These results show that the majority of the p53 PCR products analyzed indeed originate from the clonal population of HRS cells.

DISCUSSION

GC B cells are preprogrammed to die by apoptosis unless rescued by a signal through the antigen receptor.[23] HRS cells seem to be derived from GC B cells that have "crippled" their antigen receptor genes by somatic mutations.[2,3] Therefore, an intriguing question is how these cells are rescued from undergoing apoptosis. Epstein-Barr virus can immortalize B cells and in about half of the cases of HD the HRS cells are infected with this virus.[24] So far, however, there is no direct proof that apoptosis of the HRS cell precursor might be blocked by infection with Epstein-Barr virus.

A molecular feature common to many, if not all, HD cases is an abnormal accumulation of NFκB in the nuclei of HRS cells.[25,26] This might be caused by defective IκB or blocked expression of this inhibitor of NFκB.[27] Constitutive nuclear expression of NFκB has been shown to render HD-derived cell lines resistant to apoptosis-inducing stimuli and may, therefore, play a role in the rescue of crippled GC B cells from programmed cell death.[26]

The p53 gene is induced after DNA damage. Apart from arresting cells in the G1 phase of the cell cycle, p53 protein can also induce apoptosis.[28,29] To find out whether inactivation of this protein might be one of the oncogenic events that renders HRS cell precursors resistant to apoptosis, we analyzed p53 genomic sequences from single HRS cells of 8 cases of HD by a combination of micromanipulation and single-cell PCR. A

Table 4. PCR Analysis of Micromanipulated Single HRS Cells

| | HRS Cells | | | | B Cells | | | | Buffer Controls | | | |
| | | PCR Products | | | | | PCR Products | | | | PCR Products | | |
Case	Cells	E4	E5/6	E7/8	Cells	E4	E5/6	E7/8	Aliquots	E4	E5/6	E7/8
1	10	8	8	8	3	3	3	3	3	—	—	—
2	10	8	8	8	3	1	1	1	3	—	—	—
3	10	7	7	6	2	1	1	1	4	—	—	—
4	12	11	9	8	10	4	4	4	15	1	1	1
5	12	9	10	8	10	6	5	6	10	—	—	—
6	12	6	5	6	10	5	4	5	10	—	—	—
7	12	4	4	4	10	6	5	4	10	—	—	—
8	16	7	7	3	10	2	2	2	10	—	—	—

Abbreviations: E4, exon 4 of the p53 gene; E5/6, exons 5 and 6; E7/8, exons 7 and 8.

validation experiment proved the reliability of this approach. CO cells[19] were mixed with PBMC from a healthy donor and the mixture was centrifuged onto glass slides. After micromanipulation of single CO cells and B cells from immunostained cytospins, the p53 mutations known to be present in the genome of CO cells[10] were detected in all products amplified from the single CO cells but not from the control B cells. In contrast, for all 8 cases of HD analyzed, only unmutated p53 sequences were amplified from single micromanipulated HRS cells.

Several earlier studies used the SSCP technique to search for mutated p53 genomic sequences in DNA extracted from HD tumor tissue.[11-16] Mutated p53 sequences were detected in a minor fraction of cases. Chen et al,[14] followed a more refined approach. They microdissected and analyzed by PCR and SSCP areas in sections of tumor tissue that contained more than 10% HRS cells. Mutated p53 sequences were detected in 6 of 23 cases. However, the SSCP technique does not permit to attribute mutated sequences to the HRS cell population.

In 2 studies, an attempt was made to selectively analyze HRS cells for p53 mutations.[10,17,18] Gupta et al[10] enriched HRS cells by sorting large (presumably polyploid) cell nuclei from cell suspensions prepared from paraffin-embedded biopsy specimens of 2 patients. p53 genomic sequences were then amplified in a 2-rounded PCR. Sequence heterogeneities were detected by

Table 5. Coamplification of IgH Gene Rearrangements and Exons 4-8 of the p53 Gene From HRS Cells of Case 8

| | p53 | | | |
Cell*	4	5/6	7/8	V$_H$3
CD30+				
1	+	+	+	+ (clonal)
2	+	+		+ (clonal)
4	+	+		+ (unique)
5	+			+ (clonal)
6				+ (clonal)
8	+	+	+	+ (clonal)
9	+	+		+ (clonal)
12	+	+	+	
14		+		
CD20+				
36	+	+	+	
43	+	+	+	

*HRS cells were micromanipulated as large CD30+ cells, control B cells as small CD20+ cells from immunostained sections of the HD biopsy specimen.

SSCP analysis and were assumed to be due to mutations in the HRS cell population. However, direct proof for this was missing, and it was a peculiar and unexplained finding that only some of the sequences obtained from the cloned aberrantly migrating PCR products indeed harbored a mutation. In addition to the material of these 2 cases of primary HD, Gupta et al also analyzed the p53 genes of 6 HD-derived cell lines. p53 mutations were found only in cell line CO. However, in light of our earlier finding that in most, if not all cases of HD, HRS cells harbor clonal Ig gene rearrangements,[2] it is questionable whether cell line CO that carries Ig loci in germline configuration[30] is derived from HRS cells.

Trümper et al[17,18] analyzed 3 cases of HD for HRS cell-specific p53 mutations by micromanipulating single CD3-, CD20-, CD14-negative, or CD30-positive cells with HRS cell morphology from cell suspensions of fresh tumor tissues. p53 cDNA was amplified and cloned PCR products were sequenced. In each of the 3 cases, some of the cells harbored identical point mutations, whereas p53 sequences from other cells of those cases were unmutated. These data were interpreted as indication for clonal heterogeneity of HRS cells.

Our finding of only unmutated sequences in HRS cells from 8 cases is in contrast to the results of Trümper et al who found mutated p53 sequences in the majority of HRS cells in all 3 cases analyzed. We cannot explain this discrepancy. The combination of micromanipulation and single-cell PCR used in the present study has previously proven to reliably yield HRS cell-specific sequence information. In 24 of 25 cases of HD, clonal Ig gene rearrangements were obtained from HRS cells that had been identified by the same pathologist using the same histological criteria and that had been micromanipulated and analyzed exactly as in the present study (including cases 1, 2, 3, and 8 of the present study).[2,31-36] In the only case that was negative for Ig gene rearrangements, this result was later found to be because of insufficient DNA quality. For case 8 of the present study, we have provided direct evidence that the unmutated p53 sequences were indeed amplified from HRS cells and not from contaminating non-HRS cells by coamplification of the HRS clone-specific IgH gene rearrangement of this case. Our data do not rule out the possibility that in a minor fraction of HD cases, some or all HRS cells carry mutated p53 alleles. A mutation of the p53 gene present in only a minor HRS cell subclone may escape detection by analysis of a limited number of single micromanipulated HRS cells, but could

potentially be detected in SSCP-based analyses. However, if only part of the HRS cell clone carries mutated p53 alleles, these mutations must have occurred late in disease progression and are, therefore, not characteristic events that contribute to malignant transformation of HRS cell precursors in HD.

In 6 of the 8 cases analyzed, at least 80% of the HRS cells were positive for p53 by immunohistochemistry. Thus, most p53 sequences amplified from HRS cells originate from this large fraction of p53-expressing HRS cells. It has been shown that certain point mutations in exons 4 to 8 of the p53 gene can increase the half-life of p53 protein.[37] In HD, increased half-life of p53 protein was discussed as a possible reason for the increased expression levels of p53 found in HRS cells of a high proportion of cases.[38] Our data suggest that mechanisms other than mutation of the p53 gene in exons 4 to 8 must be responsible for upregulation of p53 protein in HRS cells.

ACKNOWLEDGMENT

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