Lack of Point Mutations in Exons 11–23 of the Retinoblastoma Susceptibility Gene RB-1 in Liver Metastases of Colorectal Carcinoma

B. Hildebrandt^a I. Heide^a C. Thiede^c S. Nagel^a A. Dieing^a S. Jonas^b P. Neuhaus^b
C.F. Rochlitz^e H. Riess^a A. Neubauer^d

Departments of ^aInternal Medicine I, Hematology and Oncology, and ^bDepartment of General and Transplantational Surgery, Charite, Campus Virchow Clinic, Berlin, ^cDepartment of Internal Medicine, Carl Gustav Carus University Clinic, Technical University of Dresden, and ^dDepartment of Hematology, Oncology and Immunology, Center for Internal Medicine, Philipps University, Marburg, Germany; ^eDepartment of Oncology, Kantonsspital, Basel, Switzerland

Key Words

Genes · Retinoblastoma · Tumor suppressor genes · Neoplasms · Colorectal cancer · Liver · Single-stranded conformational polymorphism

Dear Sir,

Of all solid tumors in humans the molecular carcinogenesis of colorectal cancer (CRC) is one of the best understood. However, the role of alterations of the retinoblastoma susceptibility tumor suppressor gene *RB-1* with regard to malignant transformation and metastatic progression of these neoplasms is still poorly comprehended. *RB-1* was the first tumor suppressor gene to be discovered and is located on chromosome 13q14. Its overall size amounts to 200 kb of genomic DNA, including 27 exons (4,750 bp), encoding a nuclear phosphoprotein consisting of 928 amino acids (pRB110) that can be detected in all human tissues and is phosphorylated by

activated cyclin-dependent kinases during the G1-phase. Recent data suggest that pRB110 may play an important role in switching the cell cycle towards the S-phase by releasing its covalent binding partner, transcription factor E2-F, at the restriction point [1, 2].

Inherited RB inactivation is associated with the development of retinoblastomas in children. Survivors of inherited retinoblastoma have an increased risk of developing secondary tumors, mainly osteogenic and soft tissue sarcomas. In other tumors, *RB-1* inactivation is found with varying frequency. However, there is no doubt that alterations of one or more contributors to the *RB-1*/E2F pathway (p16, cdk4, *RB-1* and E2F) play a crucial role in the majority of human malignancies. This may also apply to CRC: although so far no mutations of *RB-1* in colorectal cancer have been reported, various researchers have discussed a putative role of RB alterations in these tumors in the past [3, 4].

Evidence of the importance of RB-1 alterations in colorectal carcinogenesis was mainly provided by the finding that alterations of allelic copy numbers affecting the RB locus have been found in 30–50% of colorectal cancers. Among those, allelic am-

plifications that are specific for the genetic location of RB-1 occurred in 10%, whereas the frequency of allelic losses was not increased in most studies [5, 6]. In all CRCs which were analyzed with regard to the number of RB allelic copies, an intact RB transcript could be detected as well as an intact protein, and protein synthesis was increased in some cases. In a certain percentage of colorectal cancers one can also find an altered state of phosphorylation or increased DNA-binding capacity of pRB110, thus leading to the hypothesis of a causal connection between RB allelic multiplications and elevated pRB110 synthesis in colorectal cancer [3, 5, 7, 8]. However, this would be contradictory to previous findings describing RB-1 as a tumor suppressor gene, whose overexpression is not thought to promote malignant transformation, but should instead hamper the growth of cells.

Taken together, available data on RB alterations in colorectal cancer are somewhat confusing, and, in particular, it is still unresolved whether RB-1 exon mutations contribute to colorectal carcinogenesis at all. Remarkably, the authors of one previous study failed to demonstrate mutations of the RB

Supported in part by the 'Wilhelm Sander Stiftung'.

KARGER

Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2000 S. Karger AG, Basel
0030-2414/00/0594-0344\$17.50/0

Accessible online at:
www.karger.com/journals/ocl

Bert Hildebrandt, MD
Department of Internal Medicine, Hematology and Oncology
Charite, Campus Virchow Clinic, Augustenburger Platz 1
D-13344 Berlin (Germany)
Tel. +49 30 45053112, Fax +49 30 45053901, E-Mail bert.hildebrandt@charite.de

Fig. 1. a–c Autoradiographies of SSCP-PCR electrophoresis products of Rb exons 11–17 at 1% (**a**), 16–20 at 0.5% (**b**) and 19–23 at 1% (**c**) acrylamide concentrations.

Table 1. Patients characteristics

Total number of patients	29
<i>Demographic data</i>	
Male/female	20/9
Age, years	
Mean/median	61.3/65
Range	15–82
<i>Primary tumor site</i>	
Colon cancer	22
Rectal cancer/cancer of the rectosigmoid transition	3/1
Colorectal cancer, undetermined site	3

exon 21 (which is coding a part of the RB oncogene binding region) in 6 CRC cell lines by denaturing gel electrophoresis, although alterations in RB allelic copy number had been proven in most of those samples before. On this background, analyzing a larger number of specimens of patients with colorectal

cancer may be helpful to answer the question of whether RB-1 mutations actually play a role in colorectal carcinogenesis before further investigations may be proceeded.

We therefore performed mutational analysis of RB exons 11–23 by using a ‘single-strand conformation polymorphism (SSCP)’-analysis of PCR products (SSCP-PCR) in surgical specimens obtained from 29 patients with colorectal liver metastases, who had undergone hepatic resection between 1989 and 1993. RB exons 11–23 contain the most important protein domains (oncogene binding pockets). We chose liver metastases as an accumulation of genetic abnormalities is generally observed in advanced and metastatic tumors. Four samples of primary colorectal cancer and 2 samples of healthy colon epithelium for reference purposes were also investigated. The patients’ characteristics are summarized in table 1.

Tumor preparation, RNA isolation, and first-strand synthesis were performed as described before, as well as a standard PCR-approach of the *RB-1* gene [10, 11].

Primer sequences were:

Rb1a (5′) ATT CCT CCA CAC ACT
CCA GT
Rb 1b (5′) GAA TCA AGA TTC TGA
GAT GT (exons 11–17)
Rb 2a (5′) GGT TGT AAT GGC CAC
ATA TA
Rb 2b (5′) GAT AGG CTA GCC GAT
ACA CT (exons 16–20)
Rb 3 (5′) CCA GAC CCA GAA GCC
ATT GA
Rb 4 (5′) CCA GGA ATC CGT AAG
GGT GA (exons 19–23)

SSCP-PCR consisted of a PCR with radiolabelled [³²P]dCTP, endonuclease restriction of PCR products, high-resolution gel electrophoresis of the restriction products, and autoradiography of the electrophoresis gel. PCR was performed with cDNA of 50 ng RNA in a total volume of 10 µl, containing 200 µM dATP, dTTP, dGTP, 4 µM dCTP, 0.1 µl radiolabelled dCTP (1 µCi/µl, DuPont, Homburg, Germany), 1 × PCR buffer, 0.5 µM primer each, and 1 U Taq – polymerase (Perkin Elmer) for 30 cycles (cp. citations above) before PCR products were cleaved using appropriate restriction endo-

Fig. 2. Autoradiography of the electrophoresis gel of SSCP-PCR restriction product of exons 19–23 in samples 27 and 32. Missing bands of the large (265-bp) fragment.

nucleases (Boehringer Mannheim, Germany). In detail, we used *AccI* to cut the RB 1a/b PCR product (419 bp) into two fragments (137 and 282 bp), whereas restriction cut with *BclI* resulted in a RB2a/b PCR product (483 bp) of two (254 and 229) and those with *MaeIII* in a RB3/4 PCR product of 3 (265-, 164- and 65-bp) fragments. Gel electrophoresis (2 µl restriction product, diluted with 8 µl formamide dye) was handled vertically, using Hydrolink Long Ranger (AT-Biochem, Malvern, USA)-based electrophoresis gels at polyacrylamide concentrations of 0.5% (RB 2a/b, both fragments; RB 3/4, larger fragments) and 1% (RB1a/b, both fragments; RB 3/4, small fragment), using an electrophoresis running time of 14 h at 8 W for all samples, except for the small RB 3/4-fragment (10 h, 6 W). Autoradiography of electrophoresis gels was performed using an X-ray film sensitive to ³²P (Eastman-Kodak, New York, N.Y., USA; fig. 1).

Cycle sequencing of plasmid DNA was performed after cloning RB templates into a plasmide vector by using the TA cloning kit (Invitrogen, Groningen, The Netherlands). After ligation with the pCR I vector (Invitrogen) by a T4 ligase, transformation of plasmid into *Escherichia coli* was performed

by using 'one-shot' cells. Plasmid DNA was extracted from overnight cultures by using the QIAprep-plasmid Mini-kit (Qiagen, Hilden, Germany) before cycle sequencing (25 cycles at 96°C/30 s, 50°C/15 s, and 60°C/4 s) with 5 µl template (approximately 1 µg DNA) was initiated using the PRISM Ready reaction Dyedexoxy-Terminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany). Unincorporated redundant molecules of the dye were separated from the extension product by means of Sephadex column chromatography (Amersham, Braunschweig, Germany). Electrophoretic separation was performed in a semiautomatic sequencer under denaturing conditions on a 6% polyacrylamide gel containing urea (model 373 A, Applied Biosystems). In total, sequencing was performed in four plasmids of both of the suspicious samples, respectively, and one control probe, and each plasmid was sequenced in both orientations.

Using the SSCP-PCR approach, we found electrophoretic characteristics differing from the other samples and negative controls in 2 of the 29 samples (7%; fig. 2). These 2 samples were from liver metastases of 1 cecum and 1 sigma carcinoma. In sample 11 (patient 27; liver metastasis of a carcinoma of the sigmoid flexure) two parts and in sample 12 (patient 32; liver metastasis of a cecum carcinoma) one part of a band of the biggest fragment of RB 3/4 were missing. As cycle sequencing did not reveal RB mutations in these samples, SSCP-PCR of samples 11 and 12 was repeated, but the previously demonstrated electrophoretic mobility shifts could not be reproduced. Thus we assume that the alterations seen in the first experiment were artifacts due to temperature fluctuations between the electrophoresis gel and the glass top of the electrophoresis apparatus. This phenomenon is pre-described in the context of the separation of DNA molecules over a long distance between the two. Remarkably, both alterations were found in the 265-bp fragment of RB 3/4, which has a long running distance, indeed.

From these results we conclude that RB mutations are not a common event in ad-

vanced CRC, and thus do not play an important role in the development and metastatic progression of these neoplasms. Furthermore, these findings suggest, that the allelic changes of RB-1 in CRC described by others are not associated with mutations of functionally relevant exons.

References

- 1 Riley DJ, Lee EY-HP, Lee W-H: The retinoblastoma protein: More than a tumor suppressor. *Annu Rev Cell Biol* 1994;10:1–29.
- 2 Weinberg RA: The retinoblastoma protein and cell cycle control. *Cell* 1995;81:323–330.
- 3 Wildrick MW, Boman BM: Does the human retinoblastoma gene have a role in colon cancer? *Mol Carcinog* 1994;10:1–7.
- 4 Sellers WR, Kaelin WG Jr: Role of the retinoblastoma protein in the pathogenesis of human cancer. *J Clin Oncol* 1997;15:3301–3312.
- 5 Lothe R, Fossli T, Danielsen HE, et al: Molecular genetic studies of tumor suppressor gene regions on chromosomes 13 and 17 in colorectal tumors. *J Natl Cancer Inst* 1992;84:1100–1108.
- 6 Meling GI, Lothe RA, Borresen A-L, et al: Genetic alterations within the retinoblastoma locus in colorectal carcinomas. Relation to DNA ploidy pattern studied by cytometric analysis. *Br J Cancer* 1991;64:475–480.
- 7 Ali AA, Marcus JN, Harvey JP, et al: RB1 protein in normal and malignant human colorectal tissue and colon cancer cell lines. *FASEB J* 1993;7:931–937.
- 8 Gope R, Christensen MA, Thorson A, et al: Increased expression of retinoblastoma gene in human colorectal carcinomas relative to normal colonic mucosa. *J Natl Cancer Inst* 1990;82:310–314.
- 9 Hovig E, Smith-Sorensen B, Gebhardt MC, Ryberg D, Lothe R, Borresen A-L: No alterations in exon 21 of the RB1 gene in sarcomas and carcinomas of the breast, colon, and lung. *Genes Chromosomes Cancer* 1992;5:97–103.
- 10 Heide I, Thiede C, Sonntag T, et al: The status of p53 in the metastatic progression of colorectal cancer. *Eur J Cancer* 1997;33:1314–1322.
- 11 Neubauer A, de Kant E, Rochlitz C, et al: Altered expression of the retinoblastoma susceptibility gene in chronic lymphocytic leukemia. *Br J Hematol* 1993;85:498–503.
- 12 Hayashi K: PCR-SSCP: A simple and sensitive method for detection of mutations in the genomic DNA. *PCR Methods Appl* 1991;1:34–38.