Alterations of the retinoblastoma gene in metastatic breast cancer

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Abstract Germline mutations affecting the retinoblastoma gene (RB1) predispose to inherited retinoblastomas but also other malignancies, including breast cancer. While somatic RB1 mutations have been detected in different malignancies, information about the potential role of RB1 mutations in breast cancer is limited. Recently, we discovered RB1 mutations to be associated with resistance to anthracyclines/mitomycin in primary breast cancer. The present work is the first report evaluating RB1 mutation and epigenetic status in metastatic breast cancer. Among 148 breast cancer samples analyzed by MLPA, four samples harbored intragenic deletions/duplications: Thus, exons 1–2 were deleted in two tumors and exons 21–23 in one tumor, while one sample harbored duplication of exons 18–23. The entire RB1 gene was duplicated in two tumors and multiple amplifications were revealed in one sample. Reduced copy number was observed in 17 samples (11.5%). No point mutation or promoter hypermethylation was discovered (n = 38 and 114 tumors analyzed, respectively). Interestingly, among seven tumors expressing lack of response to epirubicin, two samples harbored alterations in RB1, contrasting none out of 16 tumors with stable disease or an objective response (P = 0.08). In summary, the frequency of RB1 alterations in metastatic lesions was not increased when compared to primary breast cancer, indicating that RB1 alterations do not play a major role in metastatic development. While a non-significant association suggesting RB1 alterations to be linked to therapy resistance was observed, our data do not suggest a major role for RB1 alterations explaining acquired drug resistance.

Keywords Breast cancer · Chemoresistance · Metastasis · Driver · Passenger · RB1

Abbreviations
LOH Loss of heterozygosity
MLPA Multiplex ligation-dependent probe amplification
PCR Polymerase chain reaction
pRb Retinoblastoma gene product
RB1 Retinoblastoma gene

Introduction

The retinoblastoma protein plays a key role regulating cell cycle [1]. RB1 (the gene coding for the retinoblastoma protein) has been found mutated in several types of cancer [2–5], and germline mutations in the RB1 gene lead to retinoblastomas, in general diagnosed within the first year of life [6].

Breast cancer is a heterogeneous category of tumors, characterized by different classes of gene expression profiles [7]. However, while hundreds of genes may be mutated, amplified or epigenetically deregulated, emerging evidence suggests a limited number of gene alterations to be responsible for malignant transformation as well as...
tumor propagation. Thus, the hypothesis defining cancer gene mutations into “drivers” versus “passengers” is gaining increasing support [8–10]. Based on this hypothesis, mutations affecting a limited number of genes (10–20 only) are considered to be of vital importance to tumor propagation [10, 11]; mutations affecting other genes are considered “passengers”, generated randomly through genetic instability without providing a growth advantage under selection “pressure” in a tumor.

Emerging evidence suggests genes for which germline mutations are associated with cancer risk (e.g., TP53, CDKN2A, and RB1) often to be somatically inactivated in sporadic tumors. While retinoblastoma survivors harboring RB1 mutations are at enhanced risk of developing breast cancer [12], the potential role of somatic RB1 gene defects in breast cancer is poorly understood. While some studies have reported loss of pRb immunostaining [13] or LOH [14] in sporadic breast cancer, little is known with respect to promoter methylation status. In a recent study, we found three point mutations and two intragenetic deletions in a total of 71 locally advanced primary breast cancers [15]. Moreover, three out of a total of four patients carrying such mutations revealed resistance towards anthracycline- or mitomycin-based chemotherapy.

Response rate to chemotherapy is lower in metastatic as compared to primary breast cancer, and metastatic disease inevitably progress towards a state of multidrug resistance [16]. Based on its key role in regulating cellular growth [1] and our recent findings of RB1 mutations associated with drug resistance [15], we postulated mutated pRb protein to be a “driver”, providing a survival advantage for RB1-mutated metastatic breast cancer cells. Thus, the aim of this study was to evaluate the incidence of RB1 genetic and epigenetic disturbances in metastatic breast cancer deposits. In addition, we correlated potential alterations to response to anthracycline containing chemotherapy.

Materials and methods

Patients

All samples were collected from a secondary, metastatic site in patients initially diagnosed with primary breast cancer; this population does not overlap with the primary breast cancer patients enrolled in our previous study [15]. Assembled information on adjuvant treatment following surgery of primary tumor, treatment of metastatic disease prior to biopsy, ER/PgR status, as well as localization of metastatic deposits is provided for the patients in the core cohort for which we performed complete gene sequencing (35 out of 38 with available records) (Table 1) with individual details for those patients harboring RB1 alterations (Table 2).

Snap-frozen tumor samples were collected from a total of 155 patients suffering from metastatic breast cancer. From this cohort, 148 samples were analyzed for gene dosage variation by MLPA, 114 samples for promoter hypermethylation, while 38 samples were screened for mutations throughout the open reading frame using RT-PCR followed by complete sequencing (core cohort). All patients gave written informed consent and the study was approved by the Regional Ethical Committee. While patients received different types of chemotherapy, RB1 status could be correlated to response to subsequent epirubicin monotherapy (applied immediately after biopsy collection) among 23 patients.

Table 1  Assembled characteristics and treatment of core cohort of patients with available records (N = 35) suffering from metastatic breast cancer included in the study

| Patient characteristics | ER/PgR status in the primary setting | ER/PgR status in the metastatic setting | Localization of metastatic deposits | Treatment |
|-------------------------|-------------------------------------|---------------------------------------|------------------------------------|-----------|
| Median age at primary breast cancer disease | ER+: 18/31 patients | ER+: 5/17 patients | ER+: 5/17 patients | Tam: 13 patients |
| Median time from primary disease to relapse | PgR+: 22/31 patients | PgR+: 6/17 patients | Unknown ER/Pgr: 18 patients | OETHO: 4 patients |
| Median time from first relapse to biopsy | PG: 37/31 patients | PG: 21/31 patients | Locoregional: 32 patients | Chemotherapy (CMF): 13 patients |
| ER/PgR status | PG: 14/31 patients | PG: 21/31 patients | Distant locations: 3 patients | Radiotherapy: 13 patients |
| Localized deposits | PG: 14/31 patients | PG: 21/31 patients | No treatment: 8 patients | No treatment: 8 patients |

CMF Cyklofosfamid/metotrexate/5-fluorouracil, Tam tamoxifen, OETHO other endocrine treatment option, FUMI 5-FU/miomiycin, Tax taxol, Xelo xeloda, AI aromatase inhibitor, ER estrogen receptor, PgR progesteron receptor
Genomic DNA purification

Genomic DNA from tumor tissue was purified using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s instructions.

RNA purification and cDNA synthesis

Total RNA was purified from snap-frozen tumor biopsies using Trizol (Life technologies) extraction and dissolved in DEPC-treated deionised water. cDNA was synthesized by reverse transcription using the Transcriptor reverse transcriptase system (Roche).

Analysis of promoter methylation

Genomic DNAs were subjected to bisulfite conversion using the CpGenome DNA Modification Kit (Intergen) according to the manufacturer’s protocol and screened by promoter methylation- and unmethylation-specific PCRs (MSP and USP).

Table 2 Characteristics and treatment of patients with RB1 alterations in breast cancer metastases

| RB1 aberrations | No of samples | Patient | Treatment | Adjuvant treatment | For metastatic disease | ER/PgR status | Localisation of metastatic deposit |
|-----------------|---------------|---------|-----------|--------------------|------------------------|---------------|-----------------------------------|
| Intragenic deletion |              |         |           |                    |                        |               |                                   |
| Exons 1–2       | 2/148 (1.4%)  | 172     | CMF       | No treatment       |                        | ER–/PgR–      | L                                 |
|                 |               | 306     | No treatment | No treatment       |                        | ER–/PgR–      | L                                 |
| Exons 21–23     | 1/148 (0.7%)  | 419     | Tam       | Adria, FUMI, Tax, Xelo |                        | ER–/PgR–      | L                                 |
| Duplication     |              |         |           |                    |                        |               |                                   |
| Exons 18–23     | 1/148 (0.7%)  | 121     | Radiotherapy, CMF | No treatment       |                        | ER–/PgR+      | L                                 |
| Exons 1–27      | 2/148 (1.4%)  | 312     | Tam, OETO | Adria, FUMI        |                        | ER+/PgR+      | Unknown                          |
|                 |               | 429     | No treatment | Tam, AI, Adria, FUMI |                        | ER+/PgR+      | L                                 |
| Multiple amplification |            |         |           |                    |                        |               |                                   |
| Exons 1–27      | 1/148 (0.7%)  | 431     | No treatment | Adria             |                        | ER–/PgR–      | Unknown                          |
| Allelic imbalance |             |         |           |                    |                        |               |                                   |
| Exons 1–27      | 17/148 (11.5%) | 314    | Radiotherapy, Tam | AI, Adria          |                        | ER+/PgR+      | Unknown                          |
|                 |               | 143     | Tam       | No treatment       |                        | ER+/PgR+      | D                                 |
|                 |               | 176     | Tam, CMF  | OETO              |                        | ER+/PgR+      | ER+/PgR+                          |
|                 |               | 389     | Tam, CMF  | Epi               |                        | ER+/PgR+      | Unknown                          |
|                 |               | 340     | No treatment | Tam             |                        | ER+           | Unknown                          |
|                 |               | 005     | Radiotherapy | Adria         |                        | ER+/PgR+      | ER+/PgR–                          |
|                 |               | 137     | Radiotherapy | Tam, AI, OETO    |                        | ER–/PgR–      | L                                 |
|                 |               | 404     | No treatment | Radiotherapy, FUMI, Epi, Tax |                        | ER+/PgR+      | L                                 |
|                 |               | 344     | Adria     | Xelo             |                        | ER–/PgR–      | Unknown                          |
|                 |               | 339     | No treatment | Tam             |                        | ER–/PgR–      | Er–/PgR–                          |
|                 |               | 122     | Radiotherapy, Tam, CMF | Tam, Adria       |                        | ER+/PgR–      | D                                 |
|                 |               | 402-1   | Tam       | Radiotherapy, AI  |                        | ER+/PgR+      | ER+/PgR–                          |
|                 |               | 315     | No treatment | No treatment     |                        | ER–/PgR–      | L                                 |
|                 |               | 305     | Tam       | No treatment     |                        | ER+/PgR+      | Unknown                          |
|                 |               | 365     | Tam       | AI, Adria, OETO  |                        | ER+/PgR+      | D                                 |
|                 |               | 182     | No treatment | No treatment     |                        | ER+/PgR+      | ER+/PgR–                          |
|                 |               | 184     | Tam       | No treatment     |                        | ER+/PgR+      | L                                 |

CMF cyclofosfamid/metotrexate/5-fluorouracil, Tam tamoxifen, OETO other endocrine treatment option, Adria Adriamycin, FUMI 5-FU/mitomycin, Tax taxol, Xelo xeloda, AI aromatase inhibitor, Epi epirubicin, ER estrogen receptor, PgR progesteron receptor, L locoregional, D distant location
Both the MSP and USP [17] were performed by an initial 5 min DNA denaturation step at 95°C followed by 35 cycles of 30 s at 94°C, 30 s at 56.6°C and 60 s at 72°C before a final elongation step at 72°C for 5 min. The 50 µl reaction solution for both PCRs consisted of 2.5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems), 1x PCR buffer, 1.5 mM MgCl2, 0.1 mM of each deoxynucleotide triphosphate, 0.2 µM of each primer (Primers are listed in Table 3), and 2 µl of modified genomic DNA.

Multiplex ligation-dependent probe amplification (MLPA)

MLPA analysis of 5 µl genomic DNA from 148 patients was performed using SALSA MLPA RB1 kit (MRC Holland, Amsterdam, The Netherlands) as previously described [15]. The peak areas achieved using RB1 specific probes in each patient sample was first normalized by the average of peak areas achieved by control probes specific for locations different from chromosome 13. A corresponding calculation was performed for two parallels of pooled DNA from 10 healthy individuals. A final ratio was then calculated by dividing the value from the patient samples by the matching value from the pool of healthy individuals. If this ratio was below 0.75, the sample was scored to have reduced copy number; If the ratio was above 1.25 the sample was scored as having increased copy number. In addition, a negative sample (H2O) was included in each run.

| RB1 exon | Sense primer | Antisense primer | Anneal. temp. (°C) |
|----------|--------------|------------------|-------------------|
| RB1 Meth | GGG AGT TTC GCG GAC GTG AC | ACG TCG AAA CAC GCC CCG | 56.6 |
| RB1 Umeth | GGG AGT TTT GTG GAT GTG AT | ACA TCA AAA CAC ACC CCA | 56.6 |
| RB1 exon 2 | GAT TAT TTT CAT TGG GTA GGC | AAA GTG GTA GGA TTA CAG GC | 52 |
| RB1 exon 3 | TTT TAA CAT AGT ATC CAG TGT GTG | TAC ACT TAC ATA ACG GCT CC | 54 |
| RB1 exon 4 | ACC CTT CGT TTT CTT ATA TTC TC | ATC AGA GTG TAA CCC TAA TAA AAT G | 55 |
| RB1 exon 5 | ATT GGG AAA ATC TAC TTG TAC | TCA AAC TAA CCC TAA TCA AG | 54 |
| RB1 exon 6 | CAT TCT ATT ATG CAT TTA ACT AAG G | CTA ACA GTT AAT AAG CCA AGC AG | 54 |
| RB1 exon 7 | ATG GAT ATA CTC TAC CCT GGC | ATC CTG TCA GCC TTA GAA CC | 55.2 |
| RB1 exon 8 | GGA TGA TAT GTG TCT TCT TAT AAT GAA ACC | GTA AAT ATT GTT AGG GAG AAC TTA CATC | 60.7 |
| RB1 exon 10 | TCT TTA ATG AAA TCT GTG CCT CTG | GAT ATC TAA AGC TCA AGC TAA AGAC | 60.7 |
| RB1 exon 11 | GAG ACA ACA GAA GCA TTA TAC TGC | TGT GAT CCA CCA CAC CTC | 60.7 |
| RB1 exon 14 | TGG GCA AAA CAG TGA GAC TCC | GAT GCC TGT ACC TCG TCA TCG G | 66.3 |
| RB1 exon 15 | TCA ATG CTG ACA CAA ATA AGG TTT C | AAA TGT TGT CAT TCA GAA GTT TGC | 60.7 |
| RB1 exon 17 | TTT TCT ATG AGT CCG TAG ACT CC | GGT AGA TGT TAA GAA ACA CCA CTC AC | 62.6 |
| RB1 exon 18 | GCC TAA AAT TCA TAC TAC TTA CCA GTT C | ATG ACT TAA TTG GGA TGA TGT TGC | 62.6 |
| RB1 exon 21 | GAT CAG TCC TGA ATA ATT GAC G | CCT ATG TTA TGT TAT GGA TAT GG | 52 |
| RB1 exon 23 | TAT GCT TCC ACC AGG GTA GGT C | GCA AAT AGA GTT TCA AGA GTC TAG CTG AT | 62.6 |
| RB1 exon 27 | CAG CCA CTT GCC AAC TTA C | CAT AAA CAG AAC CTG GGA AG | 53.5 |

1r
| RB1-1.r: A | AAC GGG AGT CCG GAG AG | GAA TTA TAT TCA CCT CTT CAT CAA G | 45.0 |
| RB1-1.r: B | ATG ATA AAA CTC TCT AGA CGT ATT C | TGT CCA CCA AGG TCG TCA G | 45.0 |

2.r
| RB1-2.r: A1 | TCG GGA GAG GAC GGC GC | CCA AGA AAC TTT TAG CAC CAA TG | 58.0 |
| RB1-2.r: A2 | AGG AGG ACC CAG AGC AAG AC | GAA GTG CAT TAG TTA CAA GTC | 53.0 |
| RB1-2.r: A3 | GTG GTA AAA GGT TCT TAC TAC | CTC TTC ATC AAG GTT AGT TCT TCG T | 58.0 |
| RB1-2.r: B1 | GAA ACA CAG AGA ACA CCA C | ATT CTG AGA TGT ACT TCT GCT A | 45.0 |
| RB1-2.r: B2 | AGC AAA CTT TGA ATG ACA AC | GAG AGG TAG ATT TCA ATG G | 45.0 |
| RB1-2.r: B3 | CTC CAA AGA AAA AAG GTT CAA | GGT ATT GGT GAC AAG GTA GG | 45.0 |
| RB1-2.r: B4 | GTA TTC TAT AAC TCG GTC TTC A | TGT CCA CCA AGG TCG TCA G | 45.0 |
two partly overlapping fragments designated A and B, covering the complete reading frame of *RB1*, were amplified under the following conditions: 94°C for 5 min, 40 cycles at 94°C for 30 s, 45°C for 30 s and 72°C for 2 min and a final extension at 72°C for 4 min. In the second round of PCR, the A and B fragments were used as templates to amplify the smaller products A1, A2, A3, B1, B2, B3 and B4. The thermocycling conditions for the second round PCRs were an initial step of 5 min denaturation at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at optimized annealing temperature and 60 s (A2, A3, B1, B2, B3, and B4) or 20 s (A1) at 72°C. As a final extension step, samples were incubated for 5 min at 72°C. All primers and annealing temperatures are listed in Table 3. Each PCR was carried out in a 50 μl reaction solution consisting of 0.5 U Dynazyme EXT DNA polymerase, 1× PCR buffer, 5% DMSO, 0.2 mM of each deoxynucleotide triphosphate, 0.2 μM of each primer, and 20 μl cDNA (first round of amplification) or 1 μl of corresponding first-round PCR product (second round of amplification).

**PCR amplification of *RB1* from genomic DNA**

PCR amplification of the *RB1* exons 2, 3, 4, 5, 6, 7, 21, and 27 from genomic DNA was carried out using an initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, optimized annealing temperature (Table 3) for 30 s and 72°C for 30 s. The final elongation was carried out at 72°C for 5 min. The PCR amplification of the exons was performed in a 50 μl reaction solution consisting of 2.5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems), 1× PCR buffer, 1.5 mM MgCl2, 0.1 mM of each deoxynucleotide triphosphate, 0.2 μM of each primer (Table 3), and 1 μl of genomic DNA.

PCR condition for amplification of the *RB1* exons 8, 10, 11, 14, 15, 17, 18, and 23 consisted of an initial 5 min denaturation at 94°C, 35 cycles of 94°C for 1 min, optimized annealing temperature (Table 3) for 5 s, and 72°C for 10 s followed by a final step at 72°C for 7 min. The reactions were carried out in a 25 μl solution containing 0.63 U KOD XL DNA polymerase (Novagen), 1× PCR buffer, 0.2 μM of each deoxynucleotide triphosphate, 0.2 μM of each primer (Table 3), and 1 μl of genomic DNA.

**TOPO-TA cloning of PCR products**

For weak PCR products resulting in non-satisfactory sequence quality, the reaction products were cloned into the pCR 2.1 TOPO vector (Invitrogen) for individual analysis. Resulting colonies were screened by amplifying the insert using M13 PCR (according to the manufacturer’s instructions). Colonies containing insert of correct length were cultured and the corresponding plasmids were purified by Qiagen spin miniprep kit (Qiagen) and sequenced. A minimum of 10 individual clones were sequenced from each sample.

**DNA sequencing**

DNA sequencing was performed directly on 1–5 μl of the PCR products or plasmids using Big Dye terminator mix (Applied Biosystems) and primers specific for each fragment. An initial denaturation at 94°C for 5 min was followed by 40 cycles of 94°C for 15 s, 50°C for 5 s and 60°C for 4 min. Capillary gel electrophoresis, data collection and sequence analysis were performed using an automated DNA sequencer (ABI 3700, Perkin–Elmer Biosystems), and the resulting sequences were compared with the *RB1* wild-type sequence (GenBank accession number L11910).

**Statistical analysis**

Statistical calculations were performed using the SPSS 15.0 software and http://www.quantitativeskills.com/sisa/. For Fisher exact tests, *P*-values are given as cumulative and two-sided.

**Results**

Complete sequencing on cDNA was performed on 38 metastatic tumor deposits (core cohort). For some of the tumor samples, it was not possible to obtain RT-PCR products for a selection of the expected fragments. Thus, we amplified the corresponding exons (2, 3, 4, 5, 6, 7, 21, and 27) from genomic DNA. The complete *RB1* reading frame excluding exon 1 was analyzed in 35 samples. As for the three remaining cases, the examination covered the complete reading frame excluding exons 1–5 due to limited tumor DNA in combination with high GC content causing technical problems. Contrasting our previous findings in non-matching primary breast cancer [15], no single point mutation was discovered in any of the metastatic samples.

Due to the potential complication of nonsense mutations and subsequent nonsense-mediated decay of *RB1* mRNA, our results were validated by analyzing exons 8, 10, 11, 14, 15, 17, 18 and 23 on genomic DNA from 28 out of 38 patients in the core cohort from whom DNA was available. These exons were analyzed since they have the highest frequencies of nonsense mutations in retinoblastoma patients ([18] and http://rb1-lovd.d-lohmann.de/). In line with our findings using RT-PCR screening, we observed no mutations in these “hot spot” exons when analyzing the same samples using genomic DNA, indicating that the likelihood of any mutation escaping our cDNA sequencing analyses are negligible.
Subsequently, the material was extended and a total of 148 samples were analyzed for larger alterations in \( RB1 \) by MLPA. We identified three tumors harboring intragenic deletions (indels) (Table 2). Thus, exons 1–2 were found to be deleted in two tumors (1.4%) and exons 21–23 deleted in one (0.7%) sample (Fig. 1). Surprisingly, we detected \( RB1 \) duplications in several tumors (Table 2). One tumor harbored a duplication of the exons 18–23, potentially rupturing the full-length pRb protein if located within the reading frame of \( RB1 \). Duplication of the entire \( RB1 \) locus was observed in two samples (1.4%), while one tumor showed multiple amplifications of the \( RB1 \) gene (0.7%). A reduction of the \( RB1 \) gene copy number (LOH for the entire locus) was observed in 17 of the samples (11.5%). This was significantly lower than the frequency previously observed by us \([15]\) in non-matching primary tumors (18/71, 25%; \( P = 0.011 \)).

Data presented in Tables 1 and 2 reveals that tumors harboring \( RB1 \) alterations do not differ from tumors in the core cohort with respect to parameters like pre-biopsy treatment, receptor status and localization of metastatic deposits.

A schematic representation of the \( RB1 \) deletions and amplifications observed by MLPA in primary breast tumors examined by us in our previous study \([15]\) and metastatic samples analyzed in this study is presented in Fig. 2.

While one of the patients with an exons 1–2 deletion and the patient harboring the exons 21–23 deletion received radiation therapy with non-evaluable response, the second patient harboring exons 1–2 deletion and the tumor containing exons 18–23 duplication both revealed lack of response to epirubicin. Thus, two out of seven tumors expressing resistance to epirubicin harbored alterations in \( RB1 \), contrasting none out of 16 tumors with stable disease or an objective response (\( P = 0.08 \), including patients analyzed by MLPA and cDNA sequencing with available clinical data on response). Interestingly, combining the data from primary breast cancer \([15]\) and the metastatic samples analyzed in this study, we find 5 out of 24 tumors lacking response to anthracycline or mitomycin therapy to
Discussion

Metastatic breast cancer inevitably progress towards multidrug resistance. Based on our recent findings of an association between anthracycline/mitomycin resistance and \( RB1 \) mutations in primary breast cancer [15], we hypothesized that \( RB1 \) mutations may act as genetic “drivers”, forcing tumor progression and therapy failure. Here, we explored the hypothesis that alterations in \( RB1 \) could be enriched in metastatic compared to primary breast cancers.

Contrary to our previous study of \( RB1 \) in non-matching primary breast cancer [15], no point mutations were observed in any of the metastatic breast cancer samples analyzed in the present work. However, two different intragenic deletions (indels) were found among three of the 148 metastatic lesions analyzed by MLPA. Our findings are not at conflict with our previous results (three point mutations and two indels in a total of 71 primary cancers), but argue against the hypothesis that \( RB1 \) mutations may act as “drivers” to the metastatic process as well as to acquired drug resistance in metastatic breast cancer.

In our previous study of \( RB1 \) in primary breast cancer [15], a total of three patients harbored a duplication of the entire \( RB1 \) gene. To the best of our knowledge, this was the first time this type of alteration was described for \( RB1 \) in breast cancer. As \( RB1 \) is a tumor suppressor, one would expect that gene duplication would have an anti-tumorigenic effect. However, the fact that the same phenomenon reappeared together with a case of multiple amplification of the complete \( RB1 \) gene (which, to our knowledge has not been described previously for \( RB1 \)), in non-matching metastatic breast lesions, indicates that duplications at the \( RB1 \) locus are not anti-tumorigenic, but rather neutral or potentially tumorigenic.

Although MLPA analysis does not provide information about the exact location of the amplified region, we assume the exons 18–23 duplication to be located intragenic, disrupting the pRb protein function. This duplication was observed in a patient expressing resistance to epirubicin. Given that this alteration could rupture the full-length pRb protein if located within the reading frame of \( RB1 \), two tumors (one harboring exons 18–23 duplication and one harboring exons 1–2 deletion) out of a total of seven tumors expressing resistance to epirubicin harbored alterations in \( RB1 \), contrasting none out of 16 tumors with stable disease or an objective response (\( P = 0.08 \)). Although the number of samples are too limited for the data to reach statistical significance, these observations are in line with our previous findings [15] suggesting that alterations in \( RB1 \) could contribute to the development of chemotherapy resistance in breast cancer.

Methylation of the \( RB1 \) promoter has been observed in various human tumors including one study in breast carcinomas [17, 19, 20]. In the present study, which is the first to analyze for promoter methylation of \( RB1 \) in metastatic breast cancer, we found no methylated tumors, indicating that methylation of the promoter region of this gene is not a key event in metastatic breast cancer. This finding is in accordance with what we have previously described for \( RB1 \) in locally advanced primary breast cancers [15].

We found the frequency of reduced \( RB1 \) gene copy number to be lower among the metastatic lesions as compared to what has been reported by others [14, 21, 22] as well as our own results reporting on non-matching primary breast cancer [15]. This may seem as a paradox, since deletion of a tumor suppressor in theory should provide cells with growth advantages and support tumor progression (“driver”). Our present finding contrasts observations in prostate carcinomas, in which the incidence of \( RB1 \) LOH was found higher in metastatic as compared to primary deposits [23]. However, our results are in line with the observations of Ellsworth et al. reporting 25% allelic imbalance for \( RB1 \) in primary breast tumor but 17% only in matched axillary lymph node metastases [24]. To the best of our knowledge, we are not aware of any other study reporting \( RB1 \) allelic imbalance status in distant metastatic breast cancer lesions. Whether reduced incidence of \( RB1 \) allelic imbalance in lymph node as well as distant metastasis indicates clonal selection or presents an acquired phenomenon [25] remains an open issue.

Taken together, we believe our studies of primary [15] and metastatic breast cancer in combination add important information in the quest to understand the role of \( RB1 \) alterations in breast cancer behavior. Although not observed in corresponding patients, the finding that the frequency of \( RB1 \) alterations did not increase from primary to metastatic lesions argues against a major role for \( RB1 \) alterations as drivers during metastatic development. On the other hand, genetic alterations disturbing \( RB1 \) function seems to be associated with resistance towards anthracycline therapy. Further studies are warranted to explore the role of other genes involved in the same pathway [26] in anthracycline resistance among primary as well as metastatic breast cancer.

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