Prognostic Significance of *ERCC1*, *RRM1* and *BRCA1* in Surgically-treated Patients with Non-small Cell Lung Cancer

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Abstract. *Chemotherapy is an important modality of treatment for non-small cell lung cancer (NSCLC). Recent studies have shown that assessment of predictive molecular markers could be helpful for estimation of the response rate to chemotherapy. The aim of our study was to assess the relation of mRNA levels of DNA repair genes excision repair cross-complementary group 1 (ERCC1), ribonucleotide reductase subunit M1 (RRM1) and breast cancer 1 (BRCA1), in surgically-resected tumor tissues from patients who underwent adjuvant chemotherapy, to the disease-free interval (DFI) and overall survival (OS). We investigated if potential residual tumor cells after resection reflect properties of the primary tumor and response to chemotherapy according to the level of predictive markers with respect to current knowledge. Patients and Methods: We studied a group of 90 patients with NSCLC who had undergone curative lung resection; 59 of them were subsequently treated with adjuvant chemotherapy, DFI and OS were evaluated only in this subgroup. Quantitative estimation of mRNA of selected genes in paired (tumor and control)-lung tissue samples was performed by real-time reverse transcription-polymerase chain reaction (RT-PCR). Results: We found a significantly lower mRNA expression of ERCC1 ($p<0.001$) and RRM1 ($p=0.023$) in NSCLC tumor tissues compared to normal lung tissues. Comparing expression in histological subtypes, we recorded higher mRNA expression of ERCC1 ($p=0.021$), RRM1 ($p=0.011$) and BRCA1 ($p=0.011$) in adenocarcinoma than in squamous cell carcinoma (SCC). Differences in DFI and OS were found only in specific subgroups according to tumor type and stage. We found longer OS for patients with adenocarcinoma with higher expression of the RRM1 mRNA ($p=0.002$), and for patients with SCC with higher expression of the BRCA1 mRNA ($p=0.041$). In patients with NSCLC of stage III, we found longer DFI in those with higher expression of RRM1 ($p=0.004$) and ERCC1 ($p=0.038$). Conclusion: Patients who had been treated with adjuvant chemotherapy and had shown lower expression of repair genes had adverse prognosis. We observed that the assessment of DNA repair gene level in primary tumors treated by surgical resection had prognostic significance and did not predict response to adjuvant chemotherapy.*

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Chemotherapy is an important modality of treatment of non-small cell lung cancer (NSCLC). Recent studies have shown that assessment of predictive molecular markers could be helpful for estimation of the response rate to chemotherapy (1-3). In early stages of NSCLC, curative treatment is performed by surgical resection. Excluding stage I disease surgical resection is usually followed by adjuvant chemotherapy (4, 5). The aim of our study was to assess the relation of mRNA levels of excision repair cross-complementary group 1 (*ERCC1*), ribonucleotide reductase subunit M1 (*RRM1*) and breast cancer-1 (*BRCA1*) in surgically-resected tumor tissues to the disease-free interval (DFI) and overall survival (OS) of patients who had undergone adjuvant chemotherapy. Furthermore, we aimed at investigating whether potential residual tumor cells after resection reflect the properties of the primary tumor and the response to chemotherapy, according to the level of predictive markers.

Despite recent advances in the treatment of NSCLC, outcomes are still unsatisfactory. Therefore a major effort of current research in NSCLC is focused on increasing treatment efficacy by using predictive molecular markers. The therapeutic benefit of chemotherapy is limited by the ability of tumor cells to overcome cytotoxicity by expression of DNA repair genes and enzymes involved in nucleic acid metabolism. *ERCC1* is a DNA damage repair gene that

Table I. *Description of the total group of patients entering the study. The distribution according to TNM, stage of disease and histology is shown.*

Group		T				N		M		Stage				Histology		
		1	2	3	4	0	≥1	0	1	I	II	III	IV	AC	SCC	Other
Men	n=65	15	38	8	4	43	22	64	1	35	14	15	1	21	41	3
Women	n=25	9	13	2	1	16	9	23	2	15	3	4	3	15	7	3
All	n=90	24	51	10	5	59	31	87	3	50	17	19	4	36	48	6
	%	27%	57%	11%	5%	66%	34%	97%	3%	55%	19%	21%	5%	40%	53%	7%

AC: Adenocarcinoma; SCC: squamous cell carcinoma.

Table II. *Description of the subgroup of patients who received adjuvant chemotherapy. The distribution according to TNM, stage of disease and histology is shown.*

Group		T				N		M		Stage				Histology		
		1	2	3	4	0	≥1	0	1	I	II	III	IV	AC	SCC	Other
Men	n=42	5	25	8	4	24	18	41	1	16	12	13	1	12	28	2
Women	n=17	4	11	1	1	9	8	15	2	8	3	3	3	11	5	1
All	n=59	9	36	9	5	33	26	56	3	24	15	16	4	23	33	3
	%	15%	61%	15%	9%	56%	44%	95%	5%	41%	25%	27%	7%	39%	56%	5%

AC: Adenocarcinoma; SCC: squamous cell carcinoma.

encodes the 5'-endonuclease of the nucleotide excision repair (NER) complex. An increase in ERCC1 expression is believed to cause the cisplatin-resistant phenotype. Cisplatin causes cytotoxicity of cancer cells by forming adducts that result in DNA cross-links. The NER complex recognizes and removes these adducts and might, thus, trigger resistance to platinum agents (6, 7).

The *RRM1* gene, located in chromosome segment 11p15.5, is a region with a frequent loss of heterozygosity in NSCLC. Its function is to convert nucleotides to deoxynucleotides. High RRM1 levels are associated with gemcitabine resistance (8, 9).

BRCA1 is a caretaker gene that encodes a pleiotropic DNA damage response protein, that functions in checkpoint activation and repairs double-strand breaks in DNA (10). Cancer cell lines deficient in BRCA1 display resistance to taxanes and are more sensitive to platinum agents (11).

Patients and Methods

Patients. The studied group consisted of 90 patients with NSCLC (median age=67.5 years, range=49-83 years, at the time of surgery) who had undergone curative lung resection at the Department of Surgery, University Hospital in Pilsen, and 59 of them were subsequently treated with adjuvant chemotherapy at the Department of Tuberculosis and Respiratory Diseases, University Hospital in Pilsen, between 2005 and 2007. The distribution according to TNM, stage of disease and histology is shown in Table I. Informed consent was received from all patients and the study was approved by the local Research Ethics Committee. Age over 85 years, other malignancy, high cardiopulmonary risk (*e.g.* chronic obstructive lung disease, condition after myocardial infarction) were considered as exclusion criteria for entering the study.

Adjuvant chemotherapy was performed according to the current guidelines of the American Society of Clinical Oncology (ASCO) 2005-2007 (12). Adjuvant chemotherapy consisted of combination of mitotic inhibitor and platinum derivative (vinorelbin plus cisplatin, or paclitaxel plus carboplatin). Out of 59 patients treated with adjuvant chemotherapy, 41 received all four recommended cycles of chemotherapy; 18 patients received less than four cycles for various reasons (unacceptable toxicity, patient refusal). Due to disease progression during the follow-up period, 15 patients were treated with palliative chemotherapy and nine patients were treated with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (erlotinib or gefitinib). No radiotherapy was applied. The median follow-up was 17.2 months.

Prognostic significance was evaluated only in 59 patients who received adjuvant chemotherapy (median age=67 years, range=49-81 years, at the time of surgery). The distribution according to TNM, stage of disease and histology is shown in Table II.

Tissue samples. Ninety paired (tumor and control) lung tissue samples were taken directly from the tumor tissue and from adjacent, histologically cancer-free lung tissue (normal lung tissue). These resected tissue samples were immediately frozen to –70˚C and stored at this temperature until usage. All the samples were histologically-verified.

Quantitative estimation of mRNA using real-time reverse transcription-polymerase chain reaction (RT-PCR). Quantitative

Table III. *Primer sequences for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with Universal Probe Library probes (Roche, Mannheim, Germany).*

Gene	Sequence of forward primer (5'-3')	UPL probe	Sequence of reverse primer (5'-3')	Amplicon length (bp)
ERCC1	GAAATTTGTGATACCCCTCGAC	79	GATCGGAATAAGGGCTTGG	66
RRM1	AAGCACCCTGACTATGCTATCC	71	GTTATAGAGGTCTTCCATCACATCAC	102
BRCA1	TTGTTGATGTGGAGGAGCAA	11	CAGATTCCAGGTAAGGGGTTC	108
GAPDH	AGCCACATCGCTCAGACAC	60	GCCCAATACGACCAAATCC	66

ERCC1: Excision repair cross-complementary group-1; *RRM1*: ribonucleotide reductase subunit M1; *BRCA1*: breast cancer-1; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

estimation of mRNA of selected genes was performed by a real-time RT-PCR method with Universal Probe Library (UPL) probes (Roche, Mannheim, Germany). UPL probes are labeled at the 5' end with fluorescein (FAM) and at the 3' end with a dark quencher dye. In order to maintain the specificity and melting temperature that hybridizing qPCR probes require, locked nucleic acids (LNAs) are incorporated into the sequence of each UPL probe. LNAs are DNA nucleotide analogs with greater binding strength compared to standard DNA nucleotides.

Total RNA was isolated from 100 mg from 90 pairs of tumor and control lung tissue (FastRNAPro Green Kit; QBIOgene, Irvine, CA, USA). Reverse transcription was performed from 500 ng of total RNA with Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) and random hexamers as primers. The sequences of primers and corresponding UPL probes were generated by ProbeFinder Software (Roche) and are shown in Table III. The primers were synthesized by GeneriBiotech (Hradec Kralove, Czech Republic). All samples were also assessed for the expression of a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The results are presented as normalized values as the ratio of the number of copies of the given gene to that of the housekeeping gene *GAPDH*.

Statistical analysis. The statistical results for comparing groups were calculated using a Wilcoxon two-sample test. *p*-Values were considered as statistically significant at the 0.05 level. Evaluation of prognostic significance was performed only for patients who had received adjuvant chemotherapy as analysis of maximum likelihood estimates (Cox model). The optimal cut-off values for the examined markers were found in the most statistically significant results (with the lowest *p*-values) of maximum likelihood estimates analysis. Kaplan-Meier survival distribution functions based on optimal cut-off values were computed for given groups.

Results

We found statistically significantly lower mRNA expression of *ERCC1* and *RRM1* in NSCLC tumor tissues in comparison with normal lung tissues ($p<0.001$ and $p=0.023$, respectively). Concerning the histological subtypes, adenocarcinoma and squamous cell carcinoma (SCC), we recorded a statistically significantly lower mRNA expression of *ERCC1* and *RRM1* in SCC tumor tissue in comparison with normal lung tissue ($p<0.001$ and $p=0.040$, respectively). Furthermore, we recorded a statistically significantly lower mRNA expression of *ERCC1* in adenocarcinoma tumor tissue in comparison with normal lung tissue ($p=0.007$).

Subsequently, we compared the expression in tumors, according to stage (I, II, III) with that of normal lung tissues. In stage I, we registered a significantly lower mRNA expression of *ERCC1* in comparison with normal lung tissue ($p<0.001$). In stage II, we found a significantly lower mRNA expression of *RRM1* and *BRCA1* in comparison with normal lung tissue ($p=0.005$ and $p=0.045$, respectively). Comparing expression the levels in different histological subtypes, we found statistically significantly higher mRNA expression of *ERCC1*, *RRM1* and *BRCA1* in adenocarcinoma than in SCC ($p=0.021$, $p=0.011$ and $p=0.011$, respectively). In stage III, no differences were statistically significant. Results of differences in expression, mentioned above, are summarized in Table IV.

We evaluated the expression level of *ERCC1*, *RRM1* and *BRCA1* in normal lung tissues in relation to adverse effects of chemotherapy (cytotoxicity). We found no relationship between expression and cytotoxicity.

In the subgroup of patients who received adjuvant chemotherapy, the data were also evaluated in relation to the DFI (in this case, time-to-recurrence of disease) and OS in NSCLC, for histological subtypes of NSCLC and also for stage groups. We found patients with adenocarcinoma, with higher expression of *RRM1* mRNA ($p=0.002$), to have a longer OS. We also recorded longer OS in patients with SCC with higher expression of *BRCA1* mRNA ($p=0.041$). In NSCLC patients with stage III disease, we found patients with higher expression of *RRM1* and *ERCC1* ($p=0.004$ and $p=0.038$, respectively) to have a longer DFI. In patients with SCC stage I, we observed longer OS in patients with higher expression of *BRCA1* and *ERCC1* ($p=0.033$ and $p=0.028$, respectively); we also found a longer DFI in patients with stage III SCC with higher expression of *ERCC1* and *RRM1* ($p=0.040$ and $p=0.036$, respectively). In patients with stage III SCC, we recorded a longer OS in those with higher expression of *RRM1* ($p=0.044$). Results on the relation of expression levels of markers to prognosis are summarized in Table V. For the markers with the strongest *p*-value, optimal

Table IV. *Differences in relative expression (housekeeping gene glyceraldehyde-3-phosphate dehydrogenase) of given markers according to tissue type and stage of the disease.*

Gene	Expression								*p*-Value
	N	25%	median	75%	N	25%	median	75%	
	Tumor				Normal lung				
ERCC1	86	0.043	0.092	0.225	85	0.139	0.259	0.707	0.001
RRM1	88	0.004	0.008	0.017	82	0.007	0.012	0.024	0.023
BRCA1	80	0	0.010	0.030	72	0.008	0.012	0.036	0.791
	Adenocarcinoma				Normal lung				
ERCC1	37	0.067	0.121	0.451	36	0.171	0.372	0.901	0.007
RRM1	36	0.005	0.010	0.024	32	0.0078	0.0136	0.0245	0.277
BRCA1	34	0.010	0.020	0.030	30	0.0096	0.0219	0.0398	0.838
	SCC				Normal lung				
ERCC1	46	0.028	0.078	0.139	45	0.113	0.214	0.500	0.001
RRM1	48	0.003	0.007	0.011	46	0.006	0.012	0.024	0.040
BRCA1	43	0.0039	0.0069	0.0199	38	0.007	0.010	0.031	0.791
	Stage I				Normal lung				
ERCC1	49	0.043	0.082	0.354	49	0.139	0.259	0.467	0.001
RRM1	48	0.005	0.009	0.019	48	0.006	0.009	0.021	0.451
BRCA1	45	0	0.010	0.030	43	0.008	0.012	0.032	0.530
	Stage II				Normal lung				
ERCC1	18	0.027	0.100	0.225	17	0.095	0.183	0.555	0.055
RRM1	18	0.007	0.009	0.014	16	0.011	0.015	0.032	0.005
BRCA1	17	0.0061	0.0099	0.0263	13	0.0087	0.0206	0.0854	0.046
	Adenocarcinoma				SCC				
ERCC1	37	0.067	0.121	0.451	46	0.028	0.078	0.139	0.021
RRM1	36	0.005	0.01	0.024	48	0.003	0.007	0.011	0.011
BRCA1	34	0.010	0.020	0.030	43	0.0039	0.0069	0.0199	0.011

ERCC1: Excision repair cross-complementary group-1; *RRM1*: ribonucleotide reductase subunit M1; *BRCA1*: breast cancer-1; SCC: squamous cell carcinoma.

cut-off values were found (Table VI). Kaplan-Meier DFI and OS curves, based on optimal cut-off values were generated (Figures 1-3).

Discussion

The predictive importance, for the better effect of chemotherapy treatment in NSCLC patients with low expression of repair genes *ERCC1*, *RRM1* and *BRCA1,* has been confirmed by many studies (9, 13, 14). However, results in published studies on the relationship of levels of these repair genes to prediction and prognosis in surgically-treated patients with NSCLC, treated with adjuvant chemotherapy are inconsistent (15-21). A recently published meta-analysis concluded that there is no difference in survival between patients with high and low tumor *ERCC1* level, who received surgery plus adjuvant chemotherapy (22).

Inconsistent results also exist regarding the comparison of the expression of DNA repair genes in tumors and normal lung tissues. In our group of patients, we recorded that the levels of *ERCC1* and RRM1 mRNA were decreased in tumor tissues in comparison with normal lung tissues of the same patients. The differences in expression of *ERCC1* in adjacent (normal) lung tissue and tumor tissue were also investigated in the work of Simon *et al*., and they found the correlation to be statistically insignificant. Simon *et al*. concluded that it is

Table V. *Relation between mRNA tumor tissue expression of given markers and disease-free interval (DFI) or overall survival (OS) in specific groups of patients with non-small cell lung cancer (Cox regression hazard model).*

Marker	Group	OS or DFI	Number of patients	β-coefficient	*p*-Value
ERCC1	SCC, stage III	OS	9	–0.344	0.04
	SCC, stage I	OS	11	–78.699	0.028
	NSCLC, stage III	DFI	14	–1.462	0.038
RRM1	Adenocarcinoma	OS	22	–0.757	0.002
	SCC, stage III	OS	10	–56.055	0.044
	NSCLC, stage III	DFI	16	–27.473	0.004
	SCC, stage III,	DFI	10	–33.840	0.036
BRCA1	SCC	OS	29	–34.743	0.041
	SCC, stage I	OS	10	–435.485	0.033

ERCC1: Excision repair cross-complementary group-1; *RRM1*: ribonucleotide reductase subunit M1; *BRCA1*: breast cancer-1; *NSCLC*: non-small cell lung cancer; SCC: squamous cell carcinoma.

Table VI. *Relation between level of given markers in tumor tissue and disease-free interval (DFI) or overall survival (OS) in specific groups of patients with non-small cell lung cancer (Kaplan-Meier estimation).*

Marker, group of patients	Number of patients	Optimal cut-off	Patients below cut-off		Patients above cut-off		*p*-Value (Mantel-Haenszel)
			N	Median (days)	N	Median (days)	
ERCC1, stage III SCC, DFI	9	0.2310	5	128	4	337	0.044
RRM1, adenocarcinoma, OS	22	0.0169	7	386	15	803	0.033
RRM1, stage III NSCLC, DFI	16	0.0085	10	144	6	643	0.033

ERCC1: Excision repair cross-complementary group-1; *RRM1*: ribonucleotide reductase subunit M1; *BRCA1*: breast cancer-1; NSCLC: non-small cell lung cancer; SCC: squamous cell carcinoma.

intratumoral *ERCC1* that is involved in tumor DNA repair and which consequently influences tumor behavior. Adjacent normal lung ERCC1 expression is, therefore, in their opinion, irrelevant (23). Jung *et al*. concluded that the expression of ERCC1 was higher in the tumor tissue than the normal tissue; however, the difference was insignificant (24). Similarly, Lenz reported that there were no differences in the expression of *ERCC1* mRNA in normal and tumor tissue (25). On the other hand, Ma *et al*. found that the level of *ERCC1* mRNA expression in cancer tissues was significantly higher than that of matched normal controls (26).

What is interesting is the difference in expression levels between SCC and adenocarcinoma tissue. We found higher expression of repair genes (*ERCC1*, *RRM1* and *BRCA1*) in adenocarcinoma. This observation supports the fact that SCC and adenocarcinoma represent tumors with different behavior.

Adjuvant chemotherapy is an important part of treatment of patients with surgically-resected NSCLC. There is a legitimate question as to what kind of cytostatic agent/chemotherapy to administer to individual patients. We dealt with the question if the assessment of expression of repair genes in the resected tissue of primary NSCLC tumor corresponds with properties of residual cells potentially surviving in the body after radical resection of the primary tumor. According to our premise, this would be reflected in the response to treatment. A patient with lower expression of DNA repair genes would respond better to chemotherapy and so would have a longer DFI or OS (8, 9). Nevertheless, we did not observe any differences in DFI or OS in relation to the expression of *ERCC1*, *RRM1* and *BRCA1* in the whole group of patients treated by surgery and adjuvant therapy. Differences were only found in specific subgroups of these patients according to tumor type and stage (SCC, stage III), where we observed that patients who had been treated with adjuvant chemotherapy and had lower expression of repair genes had an adverse prognosis.

On the basis of our result and according to studies published by other investigators (23, 27-29), we suppose that in our group of patients, those with high expression of DNA repair genes in the tumor tissue may have a better prognosis not due to the effect of adjuvant chemotherapy, but due to the absence of residual tumor cells after surgery. Or in the case of the presence of residual cells, these cells were less aggressive or their phenotype did not correspond with the phenotype of the majority of the primary tumor cells. We observed that the assessment of DNA repair gene level in

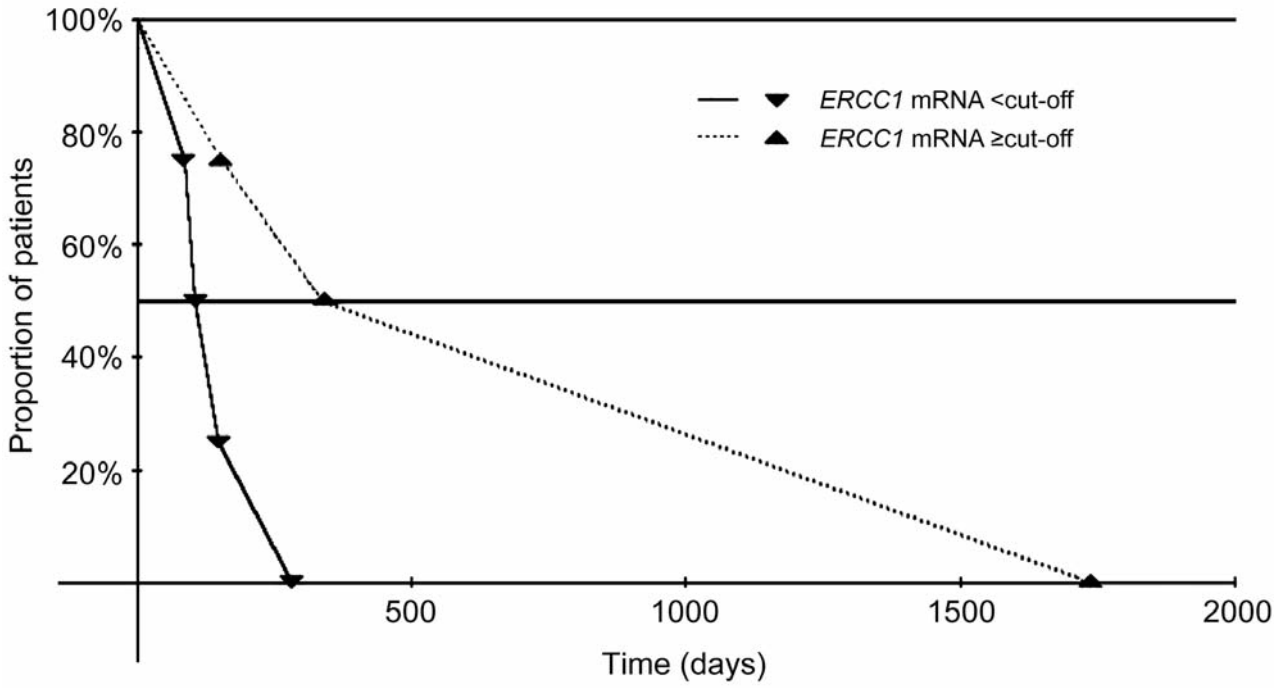

Figure 1. *Relation of excision repair cross-complementary group-1 (ERCC1) mRNA expression to overall survival (OS) in patients with stage III squamous cell carcinoma of the lung (Kaplan-Meier OS curve). There is a significant difference in the OS between patients with tissue ERCC1 expression below and above the cut-off value (p=0.044).*

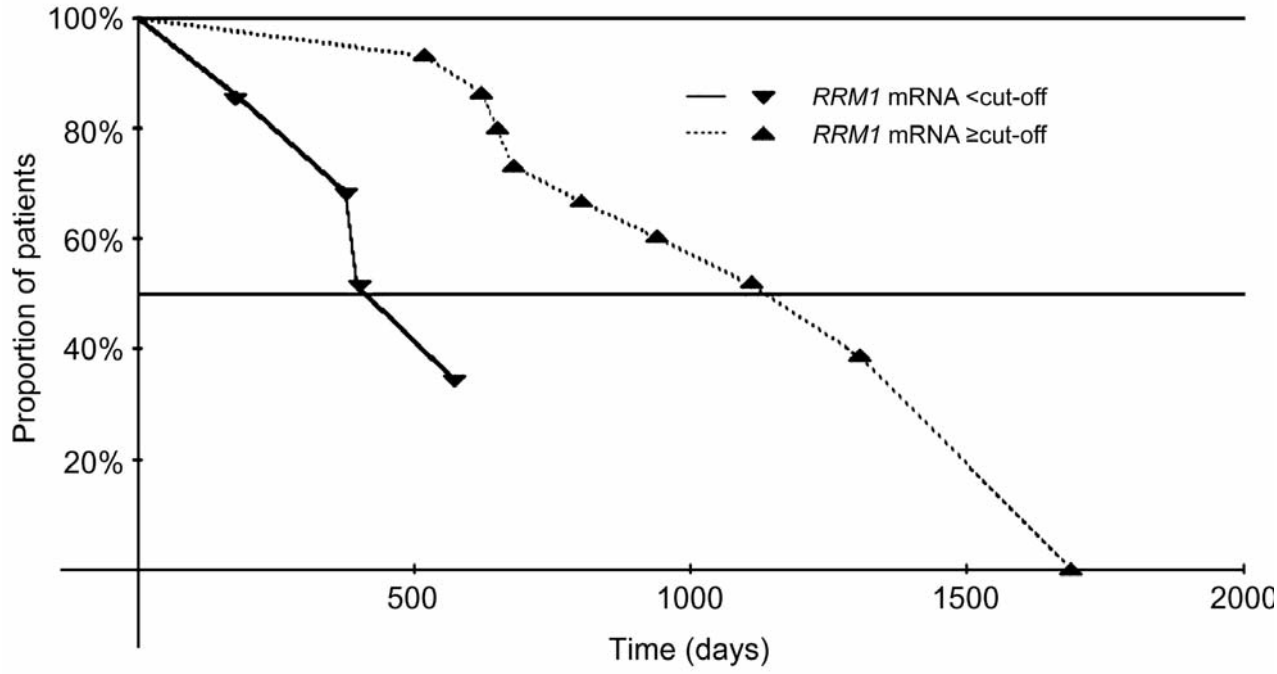

Figure 2. *Relation of ribonucleotide reductase subunit M1 (RRM1) mRNA expression to overall survival (OS) in patients with adenocarcinoma of the lung (Kaplan-Meier OS curve). There is a significant difference in the OS between patients with tissue RRM1 expression below and above the cut-off value (p=0.033).*

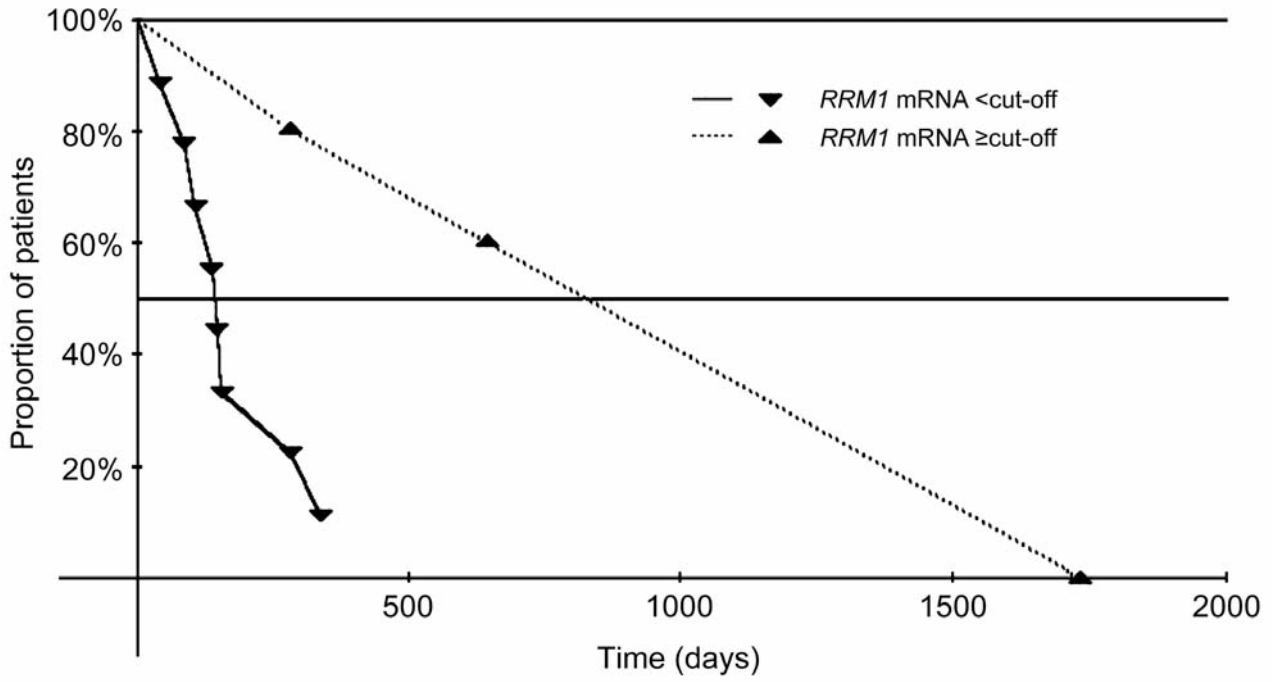

Figure 3. *Relation of ribonucleotide reductase subunit M1 (RRM1) mRNA expression to disease-free interval (DFI) in patients with stage III non-small cell lung cancer (Kaplan-Meier DFI curve). There is a significant difference in DFI between patients with tissue RRM1 expression below and above the cut-off value (p=0.033).*

primary tumor treated by surgical resection had prognostic significance and did not predict response to adjuvant chemotherapy. For *ERCC1*, a similar observation was made by Simon *et al*. (23), studying a group of surgically-treated patients; 45 patients who received no adjuvant or neoadjuvant radiation or chemotherapy, five patients who received postoperative adjuvant radiation, and one patient who received postoperative adjuvant combined radiation and chemotherapy. They postulated that an intact DNA repair mechanism may reduce the accumulation of genetic aberrations that are thought to contribute to a tumor's malignant potential and therefore reduce the risk of relapse after definitive treatment. On the other hand Bepler *et al*. observed that low *ERCC1* scores indicated significant benefit from adjuvant chemotherapy, although no other survival associations (including RRM1 protein levels) were statistically significant (21). Important work published by Olaussen *et al*. concluded that adjuvant chemotherapy, as compared with observation-alone, significantly prolonged survival among patients with ERCC1-negative tumors but not among patients with ERCC1-positive tumors (18).

Patients with early-stage disease with high repair gene expression have a good prognosis after surgery, perhaps due to the low probability of residual disease, and therefore adjuvant therapy might be unnecessary. Moreover, according to recent knowledge on the predictive significance of the expression of DNA repair genes for treatment response, for these patients, the administration of chemotherapy would be ineffective because of high repair gene expression. The estimation of predictors in primary tumor tissue could help to decide on the need for or suitability of administration of an adjuvant chemotherapy.

Conclusion

Patients with stage III SCC treated with adjuvant chemotherapy who had a lower expression of repair genes had an adverse prognosis. We found that the assessment of DNA repair gene levels in primary tumors removed by surgical resection had prognostic significance and provides information useful in the treatment decision.

Conflicts of Interest

The Authors report no conflicts of interest.

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