Altered expression of *Bcl-2*, *c-Myc*, *H-Ras*, *K-Ras*, and *N-Ras* does not influence the course of mycosis fungoides

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Abstract

Introduction: Data about genetic alterations in mycosis fungoides (MF) are limited and their significance not fully elucidated. The aim of the study was to explore the expression of various oncogenes in MF and to assess their influence on the disease course.
Material and methods: Skin biopsies from 27 MF patients (14 with early MF and 13 with advanced disease) and 8 healthy volunteers were analyzed by real-time polymerase chain reaction (PCR) to detect *Bcl-2*, *c-Myc*, *H-Ras*, *K-Ras* and *N-Ras* expression. All PCR reactions were performed using an Applied Biosystems 7900HT Fast Real-Time PCR System and interpreted using Sequence Detection Systems software which utilizes the comparative delta Ct method. The level of mRNA was normalized to GAPDH expression. All data were analyzed statistically.
Results: All evaluated oncogenes were found to be expressed in the skin from healthy controls and MF patients. *Bcl-2* (–4.2 ±2.2 vs. –2.2 ±1.1; $p = 0.01$), *H-Ras* (–3.0 ±3.3 vs. 0.6 ±2.6; $p = 0.01$) and *N-Ras* (–3.6 ±2.0 vs. –1.1 ±2.4; $p = 0.03$) were expressed at significantly lower levels in MF. No relationships between oncogene expression and disease stage, presence of distant metastases and survival were observed ($p > 0.05$ for all comparisons).
Conclusions: The pathogenic role and prognostic significance of analyzed oncogenes in MF seem to be limited and further studies are needed to establish better prognostic factors for patients suffering from MF.

Key words: cutaneous T-cell lymphoma, oncogenes, dermato-oncology, pathogenesis.

Introduction

Mycosis fungoides (MF) is a primary cutaneous lymphoma deriving from mature T-cells [1]. The natural disease course is characterized by the evolution from cutaneous patches and plaques to tumors and internal organ involvement. Genetic and molecular alterations involved in MF development and progression have not been completely elucidated to date; however, some previous studies suggested that genetic instability and gross

genomic changes may be of importance for MF pathogenesis [2, 3]. Recently, abnormal expression of several genes regulating apoptosis and cell proliferation in MF cells has been reported [4]; however, the significance of these alterations is still not fully understood. Therefore, we performed a study to further explore the gene expression of oncogenes regulating apoptosis (*Bcl-2*) and cell proliferation (*c-Myc*, *Ras* family genes) in MF in order to assess their influence on the course of the disease.

Material and methods

Twenty-seven patients with MF (14 women and 13 men) of age ranging from 41 to 93 years (mean: 62.8 ±12.9 years) were included in the study. The diagnosis of MF was based on clinical, histopathological and immunohistochemical examinations, according to the WHO-EORTC guidelines [1]. Fourteen (51.9%) patients were diagnosed with early MF (stage IA – 7, stage IB – 1, stage IIA – 6) and 13 (48.1%) with advanced MF (stage IIB – 1, stage III – 8, stage IVA – 3, stage IVB – 1). The median observation time was 6 years (range 2 to 24 years).

The control group consisted of 8 healthy volunteers (4 men and 4 women) aged between 49 and 71 years (mean: 63.3 ±8.8 years) operated on in our department for cosmetic reasons. Written informed consent was obtained from all patients and healthy volunteers. The study was approved by the Ethical Committee of Wroclaw Medical University (approval no. KB 1034/2005).

mRNA isolation and quantification

Total RNA from 100 mg of fresh frozen tissue was extracted using mirVana™ mRNA Isolation Kit (Ambion Inc., Carlsbad, CA, USA) following the manufacturer's protocol. Total RNA was transcribed with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, IN, USA) to generate cDNA for the *Bcl-2*, *c-Myc*, *H-Ras*, *K-Ras*, *N-Ras*, and *GAPDH* genes. All reactions were carried out according to the manufacturer's instructions. Briefly, the mixture containing 6 µg of total RNA, 2.5 pmol/µl of RT primer and 22.8 µl of DEPC-treated water was incubated at 65°C for 10 min, followed by RT reaction conducted using 1x RT buffer, 1 µl RNase inhibitor (40 U/µl), 4 µl dNTPs (10 mM), 2 µl DTT (0.1 M) and 2.2 µl Transcriptor High Fidelity Reverse Transcriptase (10 U/µl). cDNA synthesis was carried out at 55°C for 30 min and 5 min at 85°C to inactivate the enzyme.

Table I. Comparison of studied oncogene expression between patients with mycosis fungoides (MF) and healthy controls (results presented as $\Delta Ct = Ct_{GAPDH} - Ct_{target\ mRNA}$; *p* values according to Student's *t* test)

Parameter	Patients with MF	Healthy controls	Value of *p*
GAPDH	29.8 ±4.1*	29.8 ±3.0*	0.99
Bcl-2	–4.2 ±2.2	–2.2 ±1.1	0.01
c-Myc	–2.7 ±3.8	–3.0 ±1.8	0.83
H-Ras	–3.0 ±3.3	0.6 ±2.6	0.01
K-Ras	–6.3 ±3.7	–11.3 ±2.8	0.06
N-Ras	–3.6 ±2.0	–1.1 ±2.4	0.03

**Ct values*

The expression of *Bcl-2*, *c-Myc*, *H-Ras*, *K-Ras* and *N-Ras* was detected by real-time PCR. The total of 20 µl of reaction mixture consisted of 5 µl of cDNA, 1x TaqMan® Fast Universal PCR Master Mix and 1 µl of TaqMan PCR assays. The PCR program was initiated at 95°C for 20 s, followed by 40 thermal cycles each of 1 s at 95°C and 20 s at 60°C.

All PCR reactions were performed using an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The RT-qPCR data were interpreted using Sequence Detection Systems (SDS) software version 2.2.2, which utilizes the comparative delta Ct method. The *Ct* data were determined using default threshold settings of 0.2. The threshold number (*Ct*) is defined as the cycle number at which the fluorescence crossed the fixed threshold. The level of mRNA was normalized to GAPDH expression in each sample and presented as the ΔCt value ($\Delta Ct = Ct_{GAPDH} - Ct_{target\ mRNA}$). A lower ΔCt value referred to lower expression of target mRNA.

Statistical analysis

Pearson's χ^2 test, Student's *t* test, and Spearman's rank correlation test were used for statistical analysis where appropriate. Kaplan-Meier overall survival was calculated from the date of diagnosis until the latest follow-up or death. The differences between the curves were assessed by the log rank test. Values of *p* less than 0.05 were considered significant. Analysis was performed using Statistica® 8.0 (StatSoft, Krakow, Poland).

Results

Oncogene expression in mycosis fungoides patients and healthy controls

All evaluated oncogenes were expressed in the skin of both healthy controls and MF patients. Neither gender nor age influenced the expression levels of assessed oncogenes (data not shown). However, *Bcl-2*, *H-Ras* and *N-Ras* showed significantly lower expression in MF compared to controls (Table I). On the other hand, *K-Ras* seemed to be expressed at slightly higher levels in MF lesions than in healthy skin, but this difference did not reach statistical significance ($p = 0.06$) (Table I).

Table II. Relationship between the expression level of studied oncogenes and the prognostic parameters in mycosis fungoides (MF) (results presented as $\Delta Ct = Ct_{GAPDH} - Ct_{target\ mRNA}$; *p* values according to Student's *t* test)

Oncogene	MF stage		Value of *p*	Presence of metastases		Value of *p*
	Early	Advanced		Yes	No	
Bcl-2	–3.5 ±1.5	–5.1 ±2.6	0.06	–3.5 ±1.2	–4.4 ±2.3	0.45
c-Myc	–2.9 ±4.5	–2.4 ±3.0	0.71	–2.3 ±1.5	–2.7 ±4.1	0.85
H-Ras	–2.6 ±3.2	–3.5 ±3.4	0.47	–3.4 ±1.2	–3.0 ±3.5	0.83
K-Ras	–6.8 ±3.3	–5.9 ±4.1	0.67	–6.6 ±1.9	–6.2 ±4.1	0.88
N-Ras	–3.2 ±1.7	–4.1 ±2.2	0.3	–4.6 ±1.5	–3.4 ±2.0	0.31

Expression of oncogenes and the prognosis of mycosis fungoides

Despite some differences in the oncogene expression between MF and normal skin, no relationship was observed between the expression level of these oncogenes and disease stage (early vs. advanced MF) as well as the presence of distant metastases (Table II). Furthermore, the expression level of studied oncogenes (lower vs. higher expression than the mean value of healthy controls) did not influence patient survival (log rank test: *Bcl-2*: $p = 0.81$, *c-Myc*: $p = 0.54$, *H-Ras*: $p = 0.77$, *K-Ras*: $p = 0.45$, *N-Ras*: $p = 0.91$).

Discussion

Based on the current understanding of carcinogenesis, development of a malignancy is a multistep process involving mutations of a number of genes regulating cell death and proliferation. Over the last decade, transcriptional profiling has allowed the distinct gene expression pattern related to disease prognosis to be documented in many neoplasms, giving a hope for the development of new therapeutic strategies. Interestingly, some previous studies have also observed various genetic abnormalities in MF; however, their prognostic value has not been well established to date [2–6]. To further explore the modified gene expression profile in MF we performed a study analyzing mRNA levels of major oncogenes and their influence on the prognosis of this disease. Interestingly, we did not find a relevant association between the expression of studied oncogenes and disease course and prognosis. However, as the number of included patients was small, our results must be interpreted with caution and cannot be generalized.

Bcl-2 is an important gene involved in regulation of apoptosis. This oncogene has been shown to be abnormally expressed in various malignancies; however, studies analyzing *Bcl-2* expression in primary cutaneous T-cell lymphomas (CTCL) demonstrated conflicting results. Early studies found abundant expression of Bcl-2 protein in MF lesions [7], while Sekulovic *et al*. [4] observed that patients with advanced MF more frequently had lower expression of Bcl-2 than patients with early MF, and the decreased expression of Bcl-2 was associated with poorer outcome. Similarly, in another study on CTCL [8] the Bcl-2 expression tended to decrease with disease progression despite therapy, and the opposite was found for remissions. In the current study, the mRNA level of *Bcl-2* in MF lesional skin was significantly lower than in normal skin, but this finding was not linked with disease prognosis. Of note, Dummer *et al*. [7] also did not find any correlation between Bcl-2 expression and MF course.

Ras genes, encoding three highly homologous 21-kDa proteins, K-Ras, H-Ras and N-Ras, regulate a variety of biological processes including cell growth, differentiation, proliferation and apoptosis. The *Ras* gene family has been found to be activated in approximately 30% of human cancers, including myeloproliferative disorders and leukemias. Previous studies suggested increased expression of Ras proteins in advanced MF as compared to the early stages [9, 10]. However, Kiessling *et al*. [11] found oncogenic *Ras* mutations in only 4.4% of CTCL. All of them represented advanced disease and showed a significantly shorter survival time than patients without mutations [11]. On the other hand, Sekulovic *et al*. [4] did not find any alteration of *K-Ras* expression, similarly to our observations showing no relationship between *K-Ras* expression level and MF clinical stage and prognosis. However, as we detected *H-Ras* and *N-Ras* deregulation in MF patients compared to control counterparts, we cannot exclude the possibility that these oncogenes might be involved in MF pathogenesis, although it seems not to be of primary importance.

Finally, *c-Myc* serves as a transcription factor regulating approximately 15% of all genes in the genome, creating a target gene network involved in the cell cycle, survival, ribosome biogenesis, protein synthesis and cellular energy production. *c-Myc* deregulation has been found to be a crucial element in various malignant processes, including several solid tumors, myeloproliferative disorders and leukemias [12]. Oncogenic activity of *c-Myc* is usually triggered by chromosomal translocations, gene amplification, point mutation or viral insertion, and elevated expression is usually associated with an

unfavorable course of solid tumors [12]. Data concerning the *c-Myc* gene and protein expression in MF remain divergent. At the protein level, increased expression of c-Myc in MF was reported by Kanavaros *et al*. [13], and the level of expression was significantly higher in advanced stages than in early lesions. In contrast, Sekulovic *et al*. [4] did not find any genetic alteration of *c-Myc* expression, either in early or in advanced stages of MF. Our results seem to support these data, showing that, at least at the mRNA level, there is no alteration of *c-Myc* expression in MF.

In view of our results, further studies on the role of other oncogenes and including larger MF patient groups are needed to better understand the exact mechanism of development of this malignancy and possibly to establish new prognostic factors which might be useful for more personalized therapies in the future. Aydin *et al*. [14] suggested that Fas and p53 might be good candidates. In addition, microRNA abnormalities in MF could be an interesting alternative, as recently shown by our group [15, 16].

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