

Case Report

Genetic alterations in quadruple malignancies of a patient with multiple sclerosis: their role in malignancy development and response to therapy

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Abstract: Multiple cancers represent 2.42% of all human cancers and are mainly double or triple cancers. Many possible causes of multiple malignancies have been reported such as genetic alterations, exposure to anti-cancer chemotherapy, radiotherapy, immunosuppressive therapy and reduced immunologic response. We report a female patient with multiple sclerosis and quadruple cancers of different embryological origin. Patient was diagnosed with stage III (T3, N1a, MO) medullary thyroid carcinoma (MTC), multicentric micropapillary thyroid carcinoma, scapular and lumbar melanomas (Clark II, Breslow II), and lobular invasive breast carcinoma (T1a, NO, MO). All tumors present in our patient except micropapillary thyroid carcinomas were investigated for gene alterations known to have a key role in cancer promotion and progression. Tumor samples were screened for the *p16* alterations (loss of heterozygosity and homozygous deletions), loss of heterozygosity of *PTEN*, *p53* alterations (mutational status and loss of heterozygosity) and mutational status of *RET*, *HRAS* and *KRAS*. Each type of tumor investigated had specific pattern of analyzed genetic alterations. The most prominent genetic changes were mutual alterations in *PTEN* and *p53* tumor suppressors present in breast cancer and two melanomas. These co-alterations could be crucial for promoting development of multiple malignancies. Moreover the insertion in 4th codon of *HRAS* gene was common for all tumor types investigated. It represents frameshift mutation introducing stop codon at position 5 which prevents synthesis of a full-length protein. Since the inactivated RAS enhances sensitivity to tamoxifen and radiotherapy this genetic alteration could be considered as a good prognostic factor for this patient.

Keywords: Quadruple cancers, multiple sclerosis, *HRAS*, *PTEN*, *p53*

Background

Multiple primary malignancies represent two or more malignancies in an individual. They occur rarely in human population, representing 2.42% of all human cancers. However, their frequency have increased in recent years [1]. Multiple cancers are mainly double or triple cancers. There are less than hundred cases of quadruple cancers in the literature. There are many possible causes of multiple malignancies such as genetic alterations, exposure to anti-cancer chemotherapy, radiotherapy, immunosuppressive therapy and reduced immunological response [2].

Here we report a rare phenomenon of quadruple non synchronous malignancy in a single patient with multiple sclerosis (MS) where cancers have different embryological origin. Our patient was diagnosed with six primary malignancies but of quadruple embryological origin: medullary thyroid carcinoma (MTC), two micropapillary thyroid carcinomas, double melanoma (scapular and lumbar) and lobular invasive breast carcinoma. Medullary thyroid carcinoma, scapular and lumbar melanoma, and lobular invasive breast carcinoma were investigated for gene alterations which are known to have a key role in cancer promotion and pro-

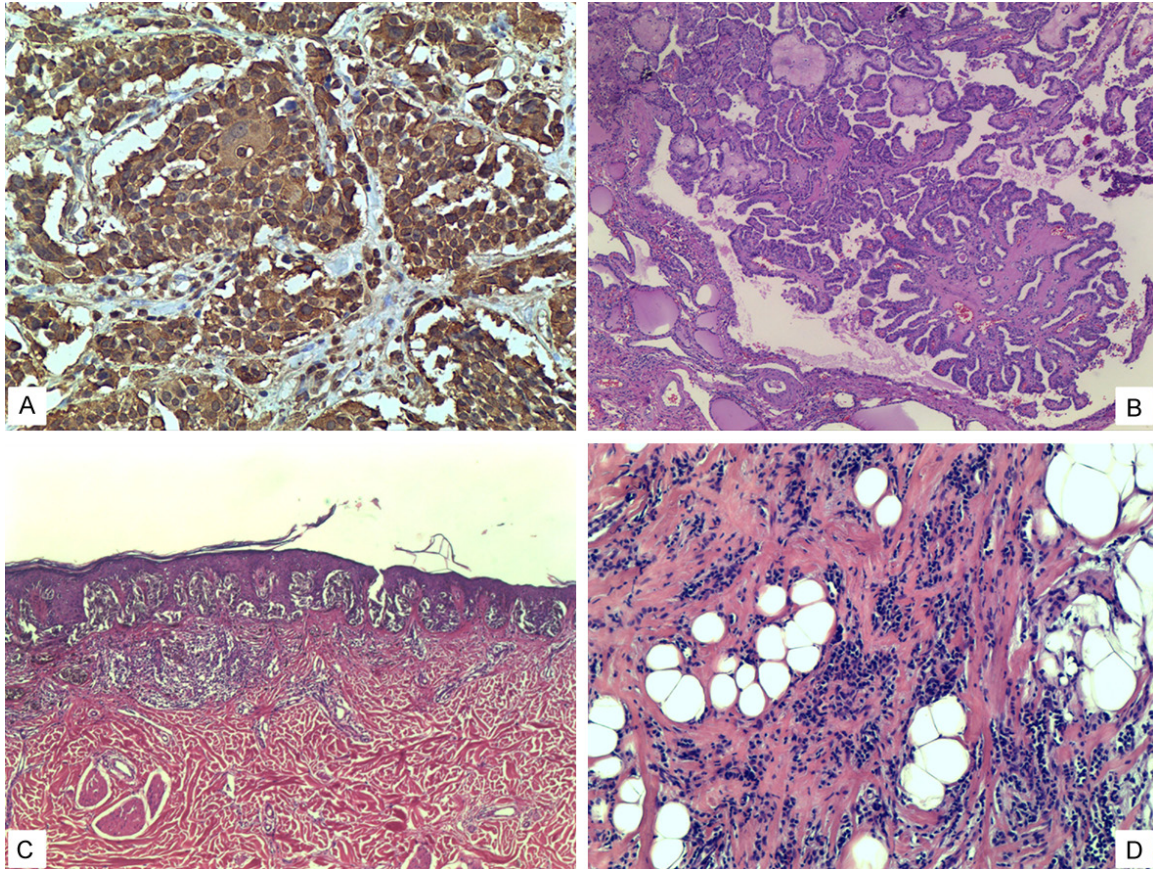


Figure 1. Hematoxylin and eosin staining (H&E). Anti-calcitonin antibody staining of medullary thyroid carcinoma (MTC) H&E x10 (A); Micropapillary thyroid carcinoma, H&E x 100 (B); Invasive cutaneous melanoma (MM 0.8 mm) H&E x10 (C); Invasive lobular breast cancer H&E x10 (D).

gression. Tumor samples were screened for the *p16* alterations (loss of heterozygosity (LOH), homozygous deletions), LOH of *PTEN*, *p53* alterations (mutational status and LOH) and mutational status of *RET* and *RAS*. *p16* and *PTEN* are tumor suppressors involved in regulation of the cell cycle, preventing cells from growing and dividing too rapidly. *p53* and *RAS* are multifunctional proteins that are critical for cell cycle regulation, apoptosis, cell survival, gene transcription, response to stress, and DNA repair [3]. *RET* proto-oncogene encodes a receptor tyrosine kinase and its gain of function mutations are associated with the development of various types of human cancer, including sporadic and hereditary medullary thyroid carcinoma [4]. The purpose of our study was to determine whether some of the detected genetic alterations are common for tumors examined, and thus could contribute to development of multiple malignancies in our patient and affect her response to therapy.

Case presentation

A 40-year-old woman was diagnosed with multiple sclerosis in 1994 and because of the severe relapsing-remitting course of the disease she was treated with pulsatile steroid therapy. Seven years later disease became secondary progressive and patient was treated with mitoxantrone (8 mg/m² intravenously every 3 months, for 15 months). There were no drug-related serious adverse events or evidence of clinically significant cardiac dysfunction. This therapy abolished the progression of disease. The patient had only moderate spastic paraparesis with gait difficulties. Expanded Disability Status Scale (EDSS) at that time was 5. Four years after mitoxantrone therapy discontinuation, in 2004, the disease exacerbated and the patient was treated with azathioprine (Aza) until 2007 when medullary thyroid carcinoma was diagnosed together with two micropapillary thyroid carcinomas. The azathio-

Table 1. Oligonucleotide Primers and PCR Conditions for *RAS* gene

RAS Gene	Exon	Primer Sequence ¹	PCR Cycling Conditions	PCR reaction mixture	
<i>K RAS</i>	<i>exon-2</i>	(a) 5'ATGACTGAATATAAACTTGT 3'	5' at 95°C; 35 cycles: 1' at 95°C, 1' at 56°C, 1' at 65°C; 7' at 65°C	MgCl ₂ dNTP	1.5 mM 2 mM
		(b) 5'CTCTATTGTTGGATCATATT 3'		Primers Taq	0.6 µm 1 U/r
<i>K RAS</i>	<i>exon-3</i>	(a) 5'AAGTAGTAATTGATGGAGAA 3'	5' at 95°C; 35 cycles: 1' at 95°C, 1' at 56°C, 1' at 65°C; 7' at 65°C	MgCl ₂ dNTP	1.5 mM 2 mM
		(b) 5'AGAAAGCCCTCCCCAGTCCT 3'		Primers Taq	0.6 µm 1 U/r
<i>H RAS</i>	<i>exon-2</i>	(a) 5'ATGACGGAACATAAGCTGGT 3'	5' at 95°C; 35 cycles: 1' at 95°C, 1' at 56°C, 1' at 65°C; 7' at 65°C	MgCl ₂ dNTP	1.5 mM 2 mM
		(b) 5'CGCCAGGCTCACCTCTATA 3'		Primers Taq	0.6 µm 1 U/r
<i>H RAS</i>	<i>exon-3</i>	(a) 5'AGGTGGTCATTGATGGGGAG 3'	5' at 95°C; 35 cycles: 1' at 95°C, 1' at 56°C, 1' at 65°C; 7' at 65°C	MgCl ₂ dNTP	1.5 mM 2 mM
		(b) 5'AGGAAGCCCTCCCCGGTGCG 3'		Primers Taq	0.6 µm 1 U/r

¹Primer (a). is the 5' primer used in the PCR and primer (b). is the 3' primer used in PCR.

prine therapy was excluded. In a period from 2007 until 2010, the patient was diagnosed with four types of tumor of different embryological origin.

In the year 2007, at the age of 53, patient was presented with a cold thyroid nodule on scintigraphy, 42 mm in diameter in the right thyroid lobe, hypoechogenic on ultrasonography, and a node of 3.3 mm in the left lobe. Serum calcitonin value was elevated over 2000 ng/L. The patient underwent total thyroidectomy, central node dissection (pretracheal and paratracheal bilateral), and sentinel lymph node biopsy of both jugular chains [5]. Patient had stage III (T3, N1a, M0) medullary thyroid carcinoma size 45 mm in the right lobe (calcitonin +, Tg -/+, CEA +, synaptophysin +, NSE +, chromogranin +, bcl2 +) (**Figure 1A**) and two micropapillary thyroid carcinomas size 1.5 and 0.5 mm (**Figure 1B**). Postoperative calcitonin value was 81.7 pg/L, CEA 2.75 ng/L and whole body I-131 scan after 24 h showed 0.93% fixation over the central neck region and no fixation over the thorax. MIBG scintigraphy was negative. Multidisciplinary committee decided a patient should be treated with 60Gy external beam irradiation (EBRT) of neck and mediastinum and L-tiroxin substitutional therapy.

A year after the thyroid surgery, in the 2008, patient complained on enlargement of pigmented skin lesion located over right scapula and other pigmented skin lesion was discovered during the clinical examination in the left lumbar region. Preoperative lymphoscintigraphy was performed and one hot node was identified in the right axilla. Both skin tumors were radically excised with more than 20 mm margins and histopathology revealed 1.24 mm

thick scapular melanoma-superficial spreading type (Clark II, Breslow II) and 0.85 mm thick lumbar melanoma-superficial spreading type (Clark II, Breslow II) with negative axillary sentinel node (**Figure 1C**).

Fourteen months later, while the perimenopausal patient was on regular follow up, the breast tumor was discovered on mammography as a stellate lesion 10.8x6.3 mm, BIRADS 5, for which she underwent quadrantectomy with level I and partially level II axillary lymph nodes dissection. Histopathology showed lobular invasive 4 mm breast carcinoma (T1a, NO, MO) LCIS (lobular carcinoma in situ) (HG II, NG II, CK7+, LCA-) (**Figure 1D**) with clear resectional margins and negative nodes (0/1). An estrogen and progesterone receptors were strongly positive and human epidermal growth factor receptor-2 overexpression was minimal. Patient underwent 40Gy postoperative radiotherapy of the left breast (extended field) considering the fact that she had been previously treated with 60Gy in the neck and mediastinum due to thyroid carcinoma. During the course of her treatment patient became menopausal and 20 mg Tamoxifen daily was added to her treatment.

FDG-PET scan performed in December 2011 was negative, CA 15-3 level was within normal values and calcitonin level was 83 ng/L and CEA 5 ng/L. Endocranial MRI indicated multiple foci of demyelination, without active lesions, and in comparison with the previous MRI findings without signs of the disease progression. Also, neurological examination performed in January 2013 did not show any signs of disease progression. She had moderate spastic paraparesis, and EDSS still 5, as it was four years ago. The patient was on regular surgical

Table 2. Mutations detected in *RET* gene

<i>RET</i> gene	Exon number	Codon number	Nucleotide change	Predicted effect
Thyroid cancer	Exon 13	769	TGC→TTC	Leu→Leu polymorphism
Thyroid cancer	Exon 16	918	ATG→ACG	Met→Thr missense

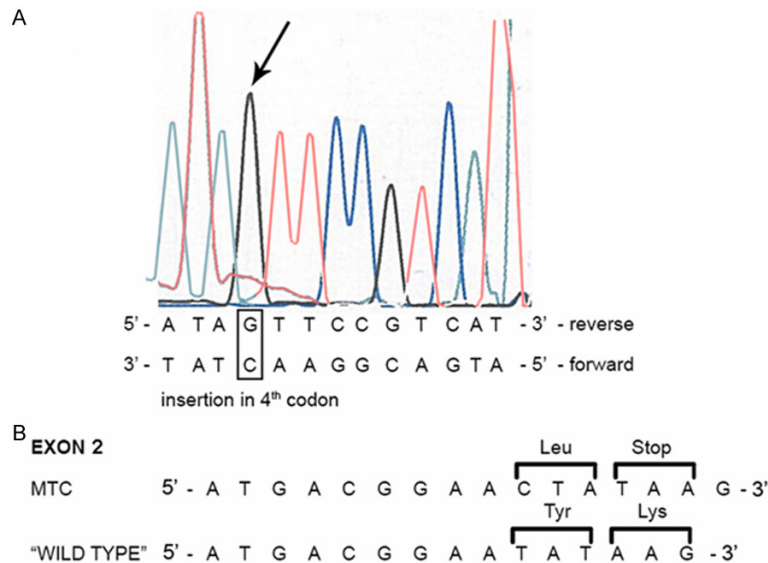


Figure 2. Representative example of DNA sequencing of exon 2 *HRAS* gene in MTC. Arrow indicates insertion of C in 4th codon detected by reverse DNA primer (upper sequence denoted as reverse) (A). Lower sequence, denoted as forward (A), represents the sequence complementary to the upper one, introduced for better illustration of the insertion and subsequent matching with the "wild-type" sequence (B).

follow up in February 2013, without signs of recurrence and with calcitonin level 40 ng/L, Tg level <0.1 ng/mL and negative Tg antibodies.

Further studies were conducted to determine genetic alterations present in four tumor samples: breast cancer, double melanoma and medullary thyroid carcinoma. DNA from paraffin-embedded tumor material was extracted using Kappa Express Extract DNA extraction kit (Kapa Biosystems, United States). DNA was isolated from patient blood as well, using Gene JET™ Genomic DNA Purification Kit (Fermentas, Lithuania) and used as a control normal tissue sample. Tumor samples were screened for the presence of *p16* LOH and homozygous deletions, LOH of *PTEN*, mutational status and LOH of *p53*, mutational status of *RAS* (*HRAS* and *KRAS*) and *RET* (only in MTC).

LOH analyses were performed using highly polymorphic microsatellite markers. Five markers spanning the *PTEN* gene (D10S579,

D10S1765, D10S215, AFM-086wg9, and D10S541) were selected to cover deletions at the whole *PTEN* locus on chromosome 10q23. All forward primers were 5'-labeled with Fam, Vic, Ned, Pet, and Fam fluorescent dyes, respectively. The microsatellite markers were chosen and locus-specific PCR performed as previously described [6]. Another set of 4 polymorphic microsatellite markers lying within or flanking *p53* gene, at the 17p13 chromosomal region, was used to examine the LOH of *p53* tumor suppressor. The chosen markers were TP53 pentanucleotide, TP53 dinucleotide, D17S1537, and D17S786 [6]. Forward primers were 5'-labeled with fluorescent dyes Fam, Pet, Ned, and Vic, respectively. Loss of hetero-

zygosity of *INK4a/ARF* locus that include *p16* tumor suppressor gene was performed using three microsatellite markers spanning the *INK4a/ARF* locus (D9S171, D9S1748 and D9S162). They were selected to cover deletions at the whole *INK4a/ARF* locus on chromosome 9p21-23. All forward primers were 5'-labeled with Ned, Pet and Vic fluorescent dyes, respectively. The microsatellite markers were chosen and locus-specific PCR performed as previously described [7]. Homozygous deletions of the *INK4a/ARF* locus, containing *p16* tumor suppressor gene, were analyzed by differential PCR. Briefly, a 199-bp fragment of *INK4a/ARF* from exon 2 was co-amplified with a 131-bp fragment of the adenine phosphoribosyltransferase (*APRT*) gene which was used as internal control. Primer sequences were described previously [7]. The PCR products for LOH and HD analysis were separated by capillary electrophoresis on an ABI Prism 3130 automated sequencer and sized using GeneScan -500 LIZ

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Table 3. Mutations detected in *KRAS* and *HRAS* genes

<i>KRAS</i> gene	Exon number	Codon number	Nucleotide change	Predicted effect
Medullary thyroid cancer	Exon 2	3	GAA→GAT	Glu→Asp missense
Breast cancer	Exon 2	5	AAA→TAA	Lys→Stop nonsense
<i>HRAS</i> gene				
Medullary thyroid cancer	Exon 2	15	GGC AAG→GCT ^a GCA	Gly Lys→Ala Ala frameshift
Medullary thyroid cancer	Exon 2	4	TAT AAG→C ^b TA TAA	Tyr Lys→Leu Stop frameshift
Lobular melanoma	Exon 2	4	TAT AAG→C ^b TA TAA	Tyr Lys→Leu Stop frameshift
Scapular melanoma	Exon 2	4	TAT AAG→C ^b TA TAA	Tyr Lys→Leu Stop frameshift
Breast cancer	Exon 2	4	TAT AAG→C ^b TA TAA	Tyr Lys→Leu Stop frameshift

^a - Insertion of CT in 15th codon; ^b - Insertion of C in 4th codon.

Table 4. Genetic alterations in four tumor samples

	<i>KRAS</i> mutations	<i>HRAS</i> mutations	LOH <i>PTEN</i>	<i>p53</i> mutations	LOH <i>p53</i>	LOH <i>p16</i>	<i>p16</i> homozygous deletion
Medullary thyroid cancer	+ ^a	+	-	-	-	-	+
Lobular melanoma	- ^b	+	+	-	+	-	-
Scapular melanoma	-	+	+	-	+	-	+
Breast cancer	+	+	+	-	+	-	+

^a - present alterations; ^b - absent alterations.

size standard (Applied Biosystems). The obtained data were analyzed with the GeneMapper software (Applied Biosystems).

Frequently mutated exons of the *p53* gene (5-9), *RET* gene (10, 11, 13, 15, 16) as well as the exons 2 and 3 (containing most frequently mutated codons 12, 13 and 61) of *RAS* gene were amplified and subjected to sequencing. Primers and PCR conditions were previously described for *p53* [6] and *RET* [8]. Primers and PCR conditions for *RAS* gene (*H-RAS* and *K-RAS*) are shown in **Table 1**. Sequences were determined with Applied Biosystems Incorporated dye terminator sequencing kit according to the manufacturer's specifications on an ABI Prism 3130 automated sequencer (Applied Biosystems, Foster City, Calif). Sequencing was carried out in both directions. The obtained sequences were analyzed and compared with wild-type *p53*, *RET* and *RAS* sequences using BLAST software in the NCBI GenBank database.

Three out of four examined tumor samples (breast cancer and two melanomas) demonstrated mutual LOH of *PTEN* and *p53*. Sequence analysis of amplified *p53* exons showed that there were no mutations present. Homozygous deletions of *INK4a/ARF* locus were present in three out of four analyzed samples (thyroid can-

cer, scapular melanoma and breast cancer). However, LOH of *p16* was not detected.

Hot spot exons of *RET* gene analyzed in medullary thyroid cancer carried two mutations. One was detected in exon 13 and the other one in exon 16. Codon positions, nucleotide changes, and predicted effects are summarized in **Table 2**. All four tumor samples had mutations in *HRAS* gene (**Figure 2**), while sequence analysis showed mutations in *KRAS* in MTC and breast cancer. The exon locations, codon positions, nucleotide changes, and predicted effects are summarized in **Table 3**. All mutations found in *KRAS* and *HRAS* were not previously reported in cancer databases.

Summary of all genetic alterations detected in tumor samples is presented in **Table 4**.

Conclusions

Multiple sclerosis has been linked to reduced rates of cancer prior to the era of immunomodulating treatments [9]. The introduction of long-term immunomodulatory treatments for MS patients has favorably affected disease course and patients' quality of life. However, patients with iatrogenic immunosuppression are more likely to develop malignancies [10]. One of immunomodulatory therapy for MS is azathioprine which was shown to confer risk of cancer

development after prolonged administration [11]. In such cases primary factor in tumor development is dysfunction of the antitumoral and antiviral properties of the immune system [10]. Therefore, taking into account prolonged treatment with azathioprine, we may assume that this drug could have contributed to development of various cancers in our patient. In this study we report various genetic changes in quadruple cancers that developed after immunosuppressive therapy with azathioprine in a female patient with MS.

Multiple malignancies isolated from our patient were analyzed for genetic alterations in *p53*, *PTEN*, and *p16* tumor suppressors, as well as *RAS* and *RET* oncogenes. The tested malignancies included medullary thyroid carcinoma, double melanoma and breast cancer. We did not perform genetic analysis of micropapillary thyroid carcinomas because the samples were too small and thus inadequate for our testing.

Medullary thyroid carcinoma had specific genetic changes in contrast to other types of tumors found in our patient. Two *RET* mutations, one in exon 13 and one in exon 16 were detected. Mutation in exon 13 in codon 769 of *RET* gene is considered as polymorphism and there is report suggesting that carriers of L769L *RET* variant do not run measurably greater risk for MTC than the general population [12]. Martin Luther University Halle-Wittenberg, Halle (Saale). Mutation in exon 16 in codon 918 is the most common mutation in patients with sporadic MTC [13]. Regarding mutational status of *RAS* gene (*HRAS* and *KRAS*) we found 3 novel mutations, two in *HRAS* and one in *KRAS* gene that were not previously reported in cancer databases. Further investigations are needed to determine the role of these mutations in medullary thyroid cancer development. Generally, previous reports described absence of *HRAS* and *KRAS* [14], as well as *NRAS* [15] mutations in sporadic MTC. *PTEN*, *p53* and *p16* LOH were not detected in MTC sample we have examined, but homozygous deletion of *INK4a/ARF* locus was present. There is evidence that *PTEN* (10q23), *p53* (17p13) and *p16/MTS-1* (9p21) are tumor suppressor genes commonly inactivated in follicular thyroid carcinomas by different mechanisms, including LOH, but very little has been done in the area of tumor suppressor genes inactivation in medullary carcinoma [16].

Both types of melanoma demonstrated LOH *p53*, LOH *PTEN* and mutation in *HRAS* oncogene. Additionally, scapular melanoma had homozygous deletion of *INK4a/ARF* locus. Breast cancer sample had alterations in all three tumor suppressors investigated, *p53*, *PTEN* and *p16* as well as mutations in *KRAS* and *HRAS* oncogenes. *HRAS* and *KRAS* mutations are rarely found in melanoma and breast cancer and therefore they present one of the specificities of our case [17, 18]. On the other hand, loss of *PTEN* has been frequently found in sporadic breast carcinoma and malignant melanoma [19, 20]. Regarding LOH of *p53* gene, it is commonly associated with breast carcinoma [21]. However, there is a very low incidence of allelic loss of this gene in melanomas [22]. As for homozygous deletion of *p16* gene, it was reported at high frequency in melanomas [23], whereas this deletion is rarely observed in primary breast cancers [24]. All tumor samples investigated had alterations in at least one tumor suppressor gene (*PTEN*, *p53* and *p16*) which indicates their importance in multiple tumor development.

Notably, concomitant LOH of *p53* and *PTEN* tumor suppressors was observed in three out of four examined tumor samples, specifically both melanomas and breast cancer. These two tumor suppressors are in complex regulatory interactions, forming positive feedback loop [25]. Their mutual inactivation is observed in many human cancers [6, 26] indicating their important role in tumorigenesis. However, such data are lacking for melanoma and the data for breast cancer are inconsistent. Unlike study of Kurose et al. where *PTEN* and *p53* mutations are mutually exclusive in human breast cancers [27], study on cell lines suggest that changes in *PTEN* and *p53* are cooperative and likely play a causal role in pathogenesis of this type of cancer [28], as we observed in our patient.

The genetic alteration present in all four tumors samples was the insertion in codon 4 of *HRAS* gene. It represents frameshift mutation introducing stop codon at position 5 which prevents synthesis of a full-length *RAS* protein. Further investigation is needed to determine whether this mutation is germline or polymorphism. In breast cancer tissue stop codon is found at position 5 of *KRAS* gene too, so this type of tumor has inactivated both *HRAS* and *KRAS* gene. The expression and activation of the

Ras/Raf-1/mitogen-activated protein kinase (MAPK) pathway plays an important role in the development and progression of cancer, and may influence response to treatments such as tamoxifen and radiotherapy. Namely, activation of the Ras pathway is responsible for poor outcome of the breast cancer patients on tamoxifen [29]. Moreover, tumor cells with endogenous RAS activation are more resistant to radiation than are their counterparts in which the mutant RAS oncogene has been inhibited [30]. The ability of the RAS oncogene to lead to radio-resistance has been indicated through application of several RAS inhibitors, such as lovastatin [31] and farnesyltransferase inhibitors [32], which block the processing of RAS and result in radio-sensitization. Since our patient responded well to tamoxifen and radiotherapy we assume that this could be due to the inactivating insertion in *HRAS* gene present in all tumor samples.

In summary, multiple malignancies in our patient with MS had numerous genetic alterations with pattern specific for each type of investigated tumor. However, we point out to the observed simultaneous alterations in *PTEN* and *p53* tumor suppressor genes present in more than one malignant tissue, specifically breast cancer and two melanomas. We assume that these co-alterations could be crucial for promoting the development of multiple malignancies in our patient. Moreover, we detected the insertion in 4th codon of *HRAS* gene common for all tumor types investigated. It represents frameshift mutation introducing stop codon at position 5 which prevents synthesis of a full-length protein. This genetic alteration could be the reason for positive therapeutic response of our patient since the inactivated RAS enhances sensitivity to tamoxifen and radiotherapy.

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Written informed consent was obtained from the patient for publication of this case report and any accompanying images.

Disclosure of conflict of interest

None.

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