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Somatic Mutations in the *RET* proto-oncogene in Sporadic Medullary Thyroid Carcinomas

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Keywords: *RET* proto-oncogene, Medullary Thyroid Carcinoma, somatic mutation, genetics, prognosis

Summary:

The frequency and prognostic relevance of *RET* proto-oncogene somatic mutations in sporadic medullary thyroid carcinoma (MTC) remain controversial. In order to study somatic mutations in the *RET* proto-oncogene in sporadic MTCs found in the Czech population and to correlate these mutations with clinical and pathological characteristics, we investigated 48 truly sporadic MTCs by sequencing classical risk exons 10, 11, 13, 14, 15 and 16. From the 48 tumors studied, 23 (48%) had somatic mutation in the *RET* proto-oncogene in exons 10, 11, 15 or 16. The classical somatic mutation Met918Thr in exon 16 was only found in 13 tumors (27%). In 5 cases, multiple somatic mutations and deletions were detected. A statistically significant correlation between the presence of somatic mutation with more advanced pathological TNM stages was observed. Other clinical and pathological characteristics did not show any statistical significant association with the presence or absence of somatic mutation.

Introduction:

Medullary thyroid carcinoma (MTC) is a malignant tumor of the neural crest-derived parafollicular C cells. It occurs in both sporadic (75%) and familial (25%) forms. The familial forms occur either as a familial MTC (FMTC) or as a part of multiple endocrine neoplasia type 2 (MEN 2) syndromes. Mutations of the *RET* proto-oncogene are involved in the pathogenesis of not only MEN 2 syndromes and FMTC, but also of sporadic MTC. Apart from germline mutations in hereditary syndromes, various somatic mutations located in the tyrosine kinase domain and cysteine-rich domain of the *RET* proto-oncogene are also found in

truly sporadic MTC. The somatic mutation Met918Thr in exon 16 in the intracellular tyrosine kinase domain as well as minor somatic mutations at codons 768 in exon 13 and 883 in exon 15 have been diagnosed in approximately one-third of sporadic MTCs (Hofstra et al. 1994, Eng et al. 1995, Marsh et al. 1996). Only a few mutations at codons 630 and 634 and deletions have been reported in the extracellular cysteine-rich domain (Romei et al. 1996, Donis-Keller et al. 1993, Hofstra et al. 1996, Ceccherini et al. 1997, Alemi et al. 1997).

The use of a detected somatic mutation as a marker of prognosis has been questioned, its value has been debated with inconclusive results in different studies. Some studies have shown a significant difference in the clinical outcome of sporadic MTC based on the presence of a codon 918 mutation (Zedenius et al. 1995, Jhiang et al. 1996, Romei et al. 1996, Wohllk et al. 1996), whereas others reported no significant difference.

In this report, we present results from the detection of somatic mutations of the *RET* proto-oncogene with genotype-phenotype correlation analysis in a cohort of 48 patients with sporadic MTCs.

Materials and Methods:

Patients

Tumor tissues were formalin-fixed and paraffin embedded (46 tissues) or fresh frozen (6 tissues). The paraffin blocks with tumor tissue were retrieved from the authors' archives (R.K., A.R. and J.D.). All patients had undergone surgery (total thyroidectomy) between 1987 and 2004.

A total of 52 apparently sporadic MTC cases were analyzed for *RET* gene mutations. These patients had no family history of hereditary MTC, pheochromocytoma, parathyroid disease, skeletal abnormalities, mucosal neuromas or Hirschsprung's disease. Table 1 describes the truly sporadic MTC patients' data.

On the basis of our genetic testing, in four cases of the 52 (7.7 %) apparently clinically sporadic MTC, germline mutations were found: in exon 10 Cys609Tyr, in exon 13 Glu768Asp, in exon 14 Val804Met and a double germline mutation in exons 10 Cys620Phe and 13 Tyr791Phe. These patients were reclassified as FMTC and excluded from our statistical analysis and descriptions.

In addition, four MEN 2 cases were included as positive controls for methodological reasons (MTC tissues obtained from two MEN 2A and two MEN 2B patients).

Clinical and pathological data

Additional clinical and pathological data was collected including sex, age at diagnosis, serum calcitonin (CT) levels after operation and at the last control (RIA kit, DFL-1200, USA; normal values were considered under 40 pg/ml and increased above 40 pg/ml), pathological TNM classification at the time of operation, presence of local and distant metastases during follow-up period, clinical outcome, length of follow-up period, disease free interval, tumor size (as the maximum diameter of the tumor), tumor differentiation (defined as well/poorly differentiated according to Shan et al. 1998), vascular invasion and the presence of amyloid and the presence of necrosis.

In the pTNM classification (accordingly to the 6th edition of AJCC - American Joint Committee on Cancer, Greene et al. 2002) the T1 category represents tumors 20 mm or less in its greatest dimension and limited to the thyroid, T2 tumors between 21 and 40 mm in its greatest dimension and limited to the thyroid, T3 tumors more than 41 mm in its greatest dimension and limited to the thyroid or with minimal extrathyroid extension and T4 tumors of any size extending beyond the thyroid capsule.

Medullary carcinomas were usually unencapsulated, showing infiltrative growth into surrounding parenchyma. They were composed mostly of polygonal and spindled cells with polymorphous nuclei, bi- or multinucleated cells were present as well.

Genetic analysis

The mutation analysis was performed with the informed consent of each patient. DNA was extracted in each case from fresh or formalin-fixed paraffin-embedded tumor tissue and corresponding non-neoplastic thyroid tissue or peripheral blood leukocytes by modified phenol-chloroform isolation protocol as described previously (Jindrichova et al. 2003). Each tumor sample included in this study was confirmed to contain a minimum of 70% tumor cells. PCR amplifications of exons 10, 11, 13, 14, 15 and 16 and subsequent double-stranded fluorescent sequencing were performed according to our previously described procedure (Jindrichova et al. 2004).

Statistical analysis:

For statistical evaluation of the data chi-squared tests, Fisher exact tests and Mann-Whitney tests were used. Values of $p < 0.05$ were considered to be statistically significant.

Results:

Frequency of somatic mutations in sporadic MTCs found in the Czech population

Germline mutations (*RET* proto-oncogene mutation detected in tumor tissue as well as in the normal control tissue or blood) were present in 4 out of 52 (7.7 %) cases of clinically apparently sporadic MTC; these cases were excluded from further analysis. Forty eight tumors carrying no germline mutation represent truly sporadic MTC. In 23 of them (48%) somatic mutations of the *RET* proto-oncogene were revealed. The detection rate and types of

somatic mutations are presented in Table 2. In 13 cases (27%) the classical somatic mutation Met918Thr in exon 16 was found. Mutations in exon 16 were also involved in another 4 cases (8%). Seven tumors carried a somatic mutation in exons 10, 11 or 15. The 6bp deletion of codons 632-633 in exon 11 was detected in 3 of these patients. Multiple mutations were found in 2 MTCs. These two cases were reported by us earlier (Dvorakova et al. 2006). In 25 samples (52%) no mutations in the six screened risk exons of the *RET* proto-oncogene were detected.

Genotype-phenotype correlation

Table 3 summarizes clinical and pathological characteristics with respect to the presence/absence of somatic mutation. As to sex distribution, there are more females in the group without somatic mutation in comparison to the group with detected somatic mutation (62.1% vs. 37.9%, Fisher exact test, n.s.). The groups did not differ in the age of the patient at diagnosis. Pathological TNM classification was significantly worse in patients with somatic mutation (T1, T2, T3 and T4 in groups with and without mutation; Fisher exact test, $p=0.022$). If T1, T2 and T3 classes were grouped and compared with the T4 class, the statistical significance was much more obvious (Fisher exact test, $p=0.003$). Interestingly, all 3 patients with the deletion in exon 11 belong to the T4 classification. Also, the proportion of patients with increased calcitonin levels after operation and at the last checkup was higher in the group with detected somatic mutation in comparison to those without mutation but the difference did not reach statistical significance (Fisher exact test, n.s.). There is a lower rate of relapse among patients who did not exhibit somatic mutation (40% vs. 60%) and these patients had a better clinical outcome in comparison to the *RET* mutation carriers (Fisher exact test, n.s.). When analyzing tumor size, tumor differentiation, invasion of vessels, presence of amyloid and/or necrosis, no statistically significant parameter was identified, but there is a trend

towards worse variants given with somatic mutations (Fisher exact test or Mann-Whitney test, n.s.).

Discussion:

The role of the *RET* proto-oncogene in the development of sporadic forms of MTC is still not fully understood. The rate of *RET* somatic mutations has been found to vary from 12% to 100% in published literature (Zedenius et al. 1994, Romei et al. 1996, Jhiang et al. 1996, Marsh et al. 1996, Marsh et al. 2003, Shan et al. 1998, Uchino et al. 1998, Uchino et al. 1999, Scurini et al. 1998, Bockhorn et al. 1999, Hofstra et al. 1994, Bugalho et al. 1997).

Several studies reported a high incidence of somatic Met918Thr *RET* proto-oncogene mutation in sporadic MTC. The frequency of this major somatic mutation has varied greatly in literature from 23% to 85% (Zedenius et al. 1994, Romei et al. 1996, Marsh et al. 1996, Shan et al. 1998, Uchino et al. 1998, Scurini et al. 1998, Bockhorn et al. 1999, Hofstra et al. 1994, Bugalho et al. 1997). However, the reason for these variations is still not clear. Some authors have suggested that the difference in frequency is due to ethnic or environmental factors or simply due to differences in detection methods.

It is hypothesized that somatic mutations of the *RET* proto-oncogene cause a continuous inappropriate activation of the RET protein, which may lead to MTC development. The Met at codon 918 is highly conserved and lies within the substrate binding pocket of the central catalytic core of the tyrosine kinase domain. Met918Thr mutation causes constitutive activation of tyrosine kinase activity as the affinity of the substrate binding pocket is altered and thus phosphorylation is increased. In fact, the mutation at codon 918 perhaps is the starting event of these neoplasms (Santoro et al. 1995, Eng et al. 1996).

The herein reported cohort is one of the largest single-country studies, we analyzed 48 truly sporadic MTC and found somatic mutations in 48% of these cases. In accordance with

other reported studies, the most common mutation was identified at codon 918 with a detection rate of 27% which represents 56.5% of all detected mutations.

In some cohorts, besides the Met918Thr mutation, other minor mutations in exons 10, 11, 12, 13 and 15 were detected (Scurini et al. 1998, Bugalho et al. 1997, Uchino et al. 1999). These somatic mutations are rarely found in patients in literature. It may be also due to the fact that not all laboratories have screened all risk exons of the *RET* proto-oncogene. We found other types of mutations (exon 16: Thr930Met, Ser922Pro, Gly911Asp, Glu921Lys; exon 11: Cys630Arg, del 6bp 632-633 codons; exon 10: Val591Ile; exon 15: Ala883Phe, Ala883Ser) in 20,8 % of the tumors.

Our mutational screening also revealed multiple mutations (exon 16: Gly911Asp+Met918Thr+Glu921Lys; exon 10+exon16: Val591Ile+Met918Thr) in 2 cases (4.2%), which were described in detail previously (Dvorakova et al. 2006). Although single somatic *RET* mutation is sufficient for MTC development, it is not clear why multiple mutations occur only in a very few cases (Marsh et al. 1996). We found no different biological or clinical features of tumors in these two cases compared to tumors with single somatic mutation. It is possible that some cell populations require additional *RET* alteration. Eng et al. 1996 and 1998 have shown previously that approximately 80% of sporadic MTCs had several subpopulations with different types of somatic *RET* mutations.

In 3 cases (6.1%) a 6bp in-frame deletion that removed codons Glu632 and Leu633 in exon 11 was identified. The three carriers of this deletion had the most aggressive tumors in our study having T4 classification. These findings are in accordance to the data reported by Bongarzone et al. 1999 where the authors described the strong transforming activity of this mutation. Deletion of codons 632-633 more effectively activated the *RET* gene in comparison to the Cys634Arg missense mutation. Deletion of codons 632-633 induced stable receptor dimer formation in the absence of a ligand. This correlated with the clinically more advanced

stage of the corresponding tumor, characterized by an unusually aggressive progression with multiple and recurrent metastases. On the other hand, Ret protein with del632-633 proceed more slowly to the cell surface than with Cys634Arg due to precursor retention in the endoplasmic reticulum.

Other types of deletions of nucleotides have been found in certain sporadic MTCs - in addition to those described above, a 3 bp deletion including codon 633, 24bp deletion including codon 634 combined with a 6bp insertion, 48bp del592-607, 27bp del611-618 or 612-620 and 12 bp del898-901 have been reported (Donis-Keller et al. 1993, Romei et al. 1996, Marsh et al. 1997, Hofstra et al. 1996, Ceccherini et al. 1997, Alemi et al. 1997, Kalinin et al. 1998, Oriola et al. 2002, Uchino et al. 1999).

Among our 48 truly sporadic MTCs, no mutations in the 6 classical risk exons were revealed in 25 of the tumors (52,1%). There are several possibilities as to the explanation of this fact: 1) mutations and/or polymorphisms able to cause MTC were present in other exons besides those we examined in this study are present. 2) factors other than somatic mutations of the *RET* proto-oncogene can play an important role in sporadic MTCs progression. Although *RET* mutation is most likely a sufficient event to cause C-cell hyperplasia, the precursor lesion to MTC, tumor progression is thought to be a result of a clonal expansion caused by the accumulation of other somatic events (Musholt et al. 2005; Montani et al. 2005; Gimm et al. 2001a, Gimm et al. 2001b). Research has found that in more than half of studied cases, chromosomal imbalances are present (Frisk et al. 2001, Marsh et al. 2003). MTC is a relatively genetically stable tumor, and chromosomal regions 19q, 19p, 13q and 11q may be involved in MTC carcinogenesis. Mutations of other candidate genes could be also involved in the pathogenesis of sporadic MTC (Musholt et al. 2005, Costa et al. 2005; Montani et al. 2005; Elisei et al. 2004; Marsh et al. 2003; Gimm et al. 2001a, Gimm et al. 2001b, Ruiz et al. 2001).

The use of detection of the somatic mutation of the *RET* proto-oncogene as a marker of prognosis has been discussed in literature. A significant correlation between the presence of somatic mutation with poor clinical outcome, distant metastasis and tumor recurrence was found, as well as concordance between positive RET immunohistochemical staining and the demonstration of the codon 918 mutation (Romei et al. 1994, Jhiang et al. 1996, Zedenius et al. 1994, Eng et al. 1998). We also found correlation between the presence of somatic mutation with more advanced pathological TNM stage. In our patients carrying somatic mutation, other trends (but not statistically significant) were apparent as well – increased CT values, worse clinical outcome and larger size of tumors with mutation. On the other hand, there are studies reporting no significant differences in parameters as the age at diagnosis, tumor size, tumor differentiation, presence or absence of metastasis, MTC-related morbidity, recurrence or prognosis base-line CT levels at diagnosis or most recent follow-up, with the presence of positive RET immunostaining between somatic mutation carriers and non-carriers (Marsh et al. 1996, Komminoth et al. 1995, Shan et al. 1998, Maeda et al. 1995, Uchino et al. 1998, Uchino et al. 1999, Scurini et al. 1998, Bockhorn et al. 1999).

These results justify the use of RET tyrosine kinase inhibitors as a promising therapy (De Groot et al. 2006). Antitumorous drugs are small tyrosine kinase inhibitors molecules that compete with ATP and thus block autophosphorylation and kinase activity and signal transduction. The most promising candidate from tested drugs seems to be anilinoquinazoline ZD6474, because it effectively blocks phosphorylation and signaling of the RET protein. Two other small tyrosine kinase inhibitors molecules, the pyrazolopyrimidine compounds PP1 and PP2, have been tested and found to be effective in therapeutic concentrations. The number of discovered small organic compounds that block RET tyrosine phosphorylation is still increasing and we hope that in the near future it can be used in the treatment of RET positive tumors and perhaps it will also be involved in a preventive strategy for treatment of patients

with somatic or germline mutations in the *RET* proto-oncogene (Carlomagno et al. 2004, Carlomagno et al. 2002, Cohen et al. 2002).

Our data suggest that the screening of tumor DNA for *RET* somatic mutations not only may help to distinguish the sporadic MTC from the familial forms with important clinical and predictive consequences, but it is also useful in the prediction of clinical outcome and therapy of certain sporadic tumors depending upon the presence and type of *RET* somatic mutation.

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Tab. 1: Detaily described patients' data

Patients	Sex	Year of operation	Age at diagnosis	Serum calcitonin level after operation	Serum calcitonin level at last control	Pathological TNM classification at operation	Disease free interval (years)	Developed distant metastasis	Developed local metastasis	Clinical outcome (disease free, high calcitonin, recurrence, died)	Somatic mutation	Tumor size (the biggest) - mm	Tumor differentiation (well x poorly differentiated)	Angioinvasion	Presence of amyloid	Necrosis
1	F	2000	47	normal	normal	T1N0M0	4	no	no	DF	Met918Thr	20	w	+	+	-
2	M	2000	49	increased	increased	T2N2M0	3	yes	yes	recurr.	Met918Thr	28	w	+	+	-
3	F	1987	42	increased	increased	T4N1M1	1	yes	yes	recurr.	Met918Thr	40	p	+	+	+
4	F	1999	64	normal	normal	T1N0M0	5	no	no	DF	Met918Thr	9	p	-	-	+
5	M	2000	50	increased	increased	T4N1M0	1	no	yes	recurr.	Met918Thr	20	w	+	+	-
6	F	2000	15	normal	normal	T2N0M0	4	no	no	DF	Met918Thr	23	p	-	-	-
7	M	1999	57	increased	increased	T4N1M0	0	yes	yes	recurr.	Met918Thr	45	p	+	+	-
8	F	2003	25	increased	increased	T3N1M0	2	yes	yes	recurr.	Met918Thr	45	w	+	+	-
9	M	2004	51	increased	increased	T1N1M0	1	no	yes	recurr.	Met918Thr	10	w	+	+	-
10	F	2000	55	normal	normal	T1N0M0	5	no	no	DF	Met918Thr	20	w	-	+	-
11	M	2002	40	increased	increased	T1N1M0	3	yes	yes	recurr.	Met918Thr	5	w	-	+	+
12	F	1999	61	increased	increased	T2N0M0	6	no	no	high CT	Met918Thr	30	w	+	+	+
13	M	1999	65	increased	increased	T4N1M0	3	yes	yes	died	Met918Thr	75	w	+	-	+
14	M	1996	18	normal	normal	T3N1M0	9	no	yes	recurr.	Gly911Asp+Met918Thr+Glu921Lys	46	w	+	+	-
15	F	2001	77	normal	normal	T1N0M0	4	no	no	DF	Val591Ile+Met918Thr	20	p	-	-	-
16	M	1998	66	increased	increased	T2N1M0	2	yes	yes	recurr.	Thr930Met	40	w	+	+	+
17	M	1999	60	increased	increased	T4N1M0	1	yes	yes	recurr.	Ser922Pro	25	p	-	-	-
18	F	2002	40	normal	increased	T1N0M0	2	no	yes	recurr.	Cys630Arg	10	w	-	+	-
19	F	2000	61	increased	increased	T4N1Mx	0.5	yes	yes	recurr.	6bp del632-633	35	w	+	+	-
20	M	2004	43	increased	increased	T4N1Mx	1	no	yes	recurr.	6bp del632-634	20	w	+	-	-
21	F	2000	59	normal	increased	T4N1MX	3	no	yes	recurr.	6bp del632-635	42	w	+	-	+
22	M	2001	76	normal	normal	T1N0MX	4	no	no	DF	Ala883Phe	20	w	-	+	+
23	M	1998	56	increased	increased	T4N1M1	1	yes	yes	recurr.	Ala883Ser	50	p	+	+	-
24	F	2001	51	normal	normal	T1N0M0	4	no	no	DF	n	10	p	-	-	-
25	F	1998	57	normal	normal	T1N0M0	7	no	no	DF	n	20	p	+	-	-
26	F	2000	60	increased	increased	T4NxMx	0	yes	yes	died	n	47	w	+	+	-
27	F	1998	48	normal	normal	T1N0M0	6	no	no	DF	n	10	w	-	+	-

28	M	1998	46	increased	increased	T3N1M1	1	yes	yes	recurr.	n	45	p	+	-	-
29	F	1999	69	increased	increased	T1N0M0	5	no	yes	recurr.	n	10	w	-	+	-
30	F	1999	46	normal	normal	T1N0M0	6	no	no	DF	n	18	w	+	+	-
31	M	2000	69	increased	increased	T1N0M0	1	yes (in 2005)	yes	recurr.	n	15	w	-	+	-
32	F	2000	71	increased	increased	T3N1M0	1	yes	yes	recurr.	n	45	p	+	-	-
33	F	1996	65	increased	increased	T2N1M1	2	yes (in 2001)	yes	recurr.	n	40	w	+	-	-
34	M	1999	41	increased	increased	T1N0M0	0.5	no	yes	recurr.	n	15	w	+	-	-
35	F	1994	64	increased	increased	T1N0M0	0	no	yes	recurr.	n	10	w	+	+	-
36	F	1995	27	increased	increased	T1N0M0	1	no	yes	recurr.	n	14	w	-	+	-
37	F	1997	54	normal	normal	T1N0M0	8	no	no	DF	n	8	w	+	+	-
38	F	1993	47	increased	increased	T1N0M0	0	yes	yes	died	n	15	p	+	+	+
39	M	1995	64	normal	normal	T1N0M0	9	no	no	DF	n	7	w	+	+	-
40	F	1995	66	normal	increased	T2N0M0	1	no	no	high CT	n	30	w	-	+	-
41	F	2002	28	increased	increased	T1N0M0	1	no	yes	recurr.	n	20	w	+	+	-
42	M	2003	59	normal	normal	T1N1M0	0	no	yes	recurr.	n	10	w	-	+	+
43	F	2004	57	normal	normal	T3N1M0	1	no	yes	recurr.	n	45	p	+	+	+
44	F	2000	71	normal	normal	T2N0M0	5	no	no	DF	n	40	w	-	-	-
45	M	1999	33	normal	normal	T1N0M0	6	no	no	DF	n	12	w	-	+	+
46	F	1998	74	increased	increased	T3N1M0	0	no	yes	recurr.	n	60	w	-	+	-
47	F	2002	80	normal	normal	T2N0M0	3	no	no	DF	n	30	p	-	-	+
48	M	2002	75	normal	increased	T1N1M0	1	yes	yes	died	n	11	p	-	+	+

Tab.2: Detection rate of the *RET* proto-oncogene somatic mutations

Type of mutation		number	%	% of mutations
exon 16	Met918Thr	13	27.1	56.5
	Thr930Met	1	2	43.4
	Ser922Pro	1	2	
	Gly911Asp, Met918Thr, Glu921Lys	1	2	
exon 10+16	Val591Ile, Met918Thr	1	2	
exon 11	Cys630Arg	1	2	
	del 6bp 632-633	3	6.1	
exon 15	Ala883Phe	1	2	
	Ala883Ser	1	2	
total with mutation		23	47.9	100
total no mutation		25	52.1	
total tumors		48	100	

Tab 3.: Clinical and pathological characteristics with respect of the *RET* somatic mutation occurrence

Characteristics		mutation (n=23)	no mutation (n=25)	P
sex	male	12 (63,1%)	7 (36,8%)	a - n.s.
	female	11 (37,9%)	18 (62,1%)	
age at diagnosis		51,17±16,21	56,88±14,60	b - n.s.
serum calcitonin level after operation	normal	9 (40,9%)	13 (59,1%)	a - n.s.
	increased	14 (53,8%)	12 (46,2%)	
serum calcitonin level at last control	normal	7 (38,9%)	11 (61,1%)	a - n.s.
	increased	16 (53,3%)	14 (46,7%)	
Pathological TNM (pTNM) classification at operation	T1	8 (33,3%)	16 (66,7%)	a - P = 0,022
	T2	4 (50%)	4 (50%)	
	T3	2 (33,3%)	4 (66,7%)	
	T4	9 (90%)	1 (10%)	
developed distant metastasis		10 (58,8%)	7 (41,2%)	a - n.s.
developed local metastasis		16 (51,6%)	15 (48,4%)	a - n.s.
clinical outcome	disease free	6 (40%)	9 (60%)	a - n.s.
	high calcitonin	1 (50%)	1 (50%)	
	recurrence	15 (55,6%)	12 (44,4%)	
	died	1 (25%)	3 (75%)	
disease free interval		2,85±2,11	2,78±2,86	b - n.s.
tumor size (mm)		29,48±16,57	23,48±15,78	b - n.s.
tumor differentiation	well	16 (48%)	17 (51,5%)	a - n.s.
	poorly	7 (46,7%)	8 (53,3%)	
presence of vascular invasion		15 (53,6%)	13 (46,4%)	a - n.s.
presence of amyloid		16 (48,5%)	17 (51,5%)	a - n.s.
presence of necrosis		8 (57,1%)	6 (42,9%)	a - n.s.

a - Fisher exact test was used

b - Mann Whitney test was used