Circulating Tyrosinase and MART-1 mRNA does not Independently Predict Relapse or Survival in Patients with AJCC Stage I–II Melanoma

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The detection of melanoma cells in peripheral blood has been proposed to select patients with a high risk of relapse. In this study, tyrosinase and melanoma antigen recognized by T cells 1 (MART-1) mRNA expression was evaluated in serial samples obtained before definitive surgery and during follow-up in patients with American Joint Committee on Cancer stage I–II melanoma. Serial samples ($n=2,262$) were collected from 236 patients from 1997 to 2002. Analyses of the RNA samples were performed with a calibrated reverse transcriptase-PCR assay. Gender, age, primary tumor site, ulceration, thickness, Clark level, and histological subtype were analyzed together with tyrosinase and MART-1 mRNA treated as updated covariates in a Cox proportional-hazard model. After a median follow-up time of 66 months, 42 out of 236 patients (18%) had relapsed. The following variables were significantly associated with relapse-free survival in the univariate analyses: tyrosinase, MART-1, gender, ulceration, thickness, Clark level, and histological subtype. Entering these covariates into a multivariate Cox analysis resulted in thickness as the single independent prognostic factor ($P<0.0001$), whereas MART-1 ($P=0.07$) approached significance at the 5% significance level. The serial measurements of tyrosinase and MART-1 mRNA in peripheral blood of stage I–II melanoma patients cannot be demonstrated to have independent prognostic impact on relapse-free survival.

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INTRODUCTION

The detection of circulating melanoma cells with reverse transcriptase (RT)-PCR has been proposed as an effective tool in selecting patients with a high risk of relapse before enrolment in adjuvant treatment studies (Curry *et al.*, 1998; Ghossein *et al.*, 1998).

Tyrosinase and and melanoma antigen recognized by T cells 1 (MART-1) are two of the most specific proteins involved in melanocytic differentiation (Battyani *et al.*, 1993; Kawakami *et al.*, 1994) and the detection of tyrosinase and MART-1 mRNA in peripheral blood have been evaluated in a number of studies (Hoon *et al.*, 1995; Palmieri *et al.*, 1999; Keilholz *et al.*, 2004; Schmidt *et al.*, 2005). The majority of these studies have investigated American Joint Committee on Cancer (AJCC) stage III–IV patients and only few patients with stage I–II melanoma have been followed with serial blood samples for a sufficient follow-up time. Thus, we know little about the prognostic impact of tyrosinase and MART-1 mRNA in peripheral blood of stage II patients and even less in stage I patients. The meta-analysis by Tsao *et al.* (2001) suggested that tyrosinase mRNA was of limited value given the unreliability of the procedure and the lack of sufficient follow-up in studies with stage I–III patients. In a recent meta-analysis, specifically addressing the relationship between serial blood samples during follow-up in relation to relapse, it was concluded that tyrosinase mRNA was a potentially useful marker, at least in the follow-up of stage III patients (Quaglino *et al.*, 2004).

In the current study, we examined serial blood samples for circulating melanoma cells, based on tyrosinase and MART-1 mRNA expression, from the time of definitive surgery of the primary lesion and during follow-up in 236 consecutive stage I–II melanoma patients.

RESULTS

This study was conducted on serial blood samples from 236 stage I–II melanoma patients. A total of 2,262 serum samples were collected before definitive surgery and during the follow-up period. The median number of samples collected from each patient was 10, with a range from 1 to 15 samples (one sample, 4%; two to seven samples, 14%; eight to ten

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Abbreviations: AJCC, American Joint Committee on Cancer; HR, hazard ratio; RT, reverse transcriptase

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Table 1. Demographic and pathologic data for 236 patients with AJCC stage I–II melanoma

	AJCC stage					
	I (*n*=164)		II (*n*=72)		Total (*n*=236)	
	No.	%	No.	%	No.	%
Gender						
Female	93	57	50	69	115	49
Male	71	43	22	31	121	51
Age (years)						
Median	52		60		56	
Range	25–81		25–86		25–86	
Ulceration						
None	160	98	35	49	195	83
Present	4	2	37	51	41	17
Histological subtype						
SSM	150	91	26	36	176	75
Others	14	9	45	63	59	25
Missing[1]	0	0	1	0.4	1	0.4
Thickness (mm)						
Median	0.8		2.8		1.1	
Range	0.2–2.0		1.1–21.0		0.2–21.0	
Missing[1]	0	0	12	17	12	5
Clark level						
Level I–III	144	88	21	29	165	70
Level IV–V	20	12	41	57	61	26
Missing[1]	0	0	10	14	10	4
Primary tumour site						
Head and trunk	95	58	44	61	139	59
Limbs	69	42	28	39	97	41

AJCC, American Joint Committee on Cancer version 2001; SSM, superficial spreading melanoma.
[1]Missing, tumours with an incomplete histopathological report.

samples, 38%; 11 to 15 samples, 44%). The median follow-up interval between the first sample and the last follow-up or death was 66 months (range 1–97 months). The median follow-up interval from the first to the last blood sample was 36 months (range 1–66 months). The patients' characteristics are summarized in Table 1.

Tyrosinase and MART-1

Tyrosinase and MART-1 was observed in 111 (5%) and 172 samples (7%), respectively. Twenty-nine patients (12%) had a positive determination of tyrosinase or MART-1 before definitive surgery. Of these, 20 patients were stage I and nine patients stage II. During the follow-up time, 142 patients (60%) had at least one positive determination of tyrosinase or MART-1. In Figure 1 the percentage of positive tyrosinase and MART-1 samples are shown as a function of time. The proportion of patients with a positive sample during the follow-up period was similar between stages IA and IIB (Table 2). No significant associations or correlations were observed between a positive baseline sample and gender, age, ulceration, primary tumor site, thickness, Clark level, and histological subtype. No correlations were observed for patients with a positive follow-up sample and the above-mentioned baseline risk factors. Control samples were obtained from 89 individuals with nevi and non-melanoma cancers. None of these samples were positive for tyrosinase. However, two patients with nevi had a positive MART-1 analysis.

Association of serial tyrosinase and MART-1 and relapse-free survival

Relapses during the follow-up time were observed in 42 out of 236 patients (18%). One-half of the patients developed distant metastases as their first relapse and the other half loco-regional relapse. Of the 42 relapsing patients, no samples were positive in 25 patients (60%), in 12 patients (29%) at least one sample was positive more than 6 months before relapse, and finally in five patients (11%) at least one sample was positive within 6 months before relapse. Follow-up samples were obtained after surgical excision of a local relapse in seven patients. In three of these patients, positive samples were also seen after surgery.

In the univariate analyses, the following variables were significantly ($P<0.10$) associated with relapse-free survival: tyrosinase mRNA, MART-1 mRNA, gender, ulceration, thickness, Clark level, and histological subtype (Table 3). Tyrosinase and MART-1 (negative *versus* positive) were both considered as updated covariates owing to the serial measurements. Entering these covariates into a multivariate Cox analysis with backward selection resulted in thickness as an independent prognostic factor (hazard ratio (HR) 4.1, 95% confidence interval (CI), 2.7–6.2; $P<0.0001$). MART-1 (HR 3.1, 95% CI, 0.9–10.2; $P=0.07$) was close to achieving significance at the 5% significance level, whereas tyrosinase (HR 2.7, 95% CI, 0.4–16.7; $P=0.36$) was not significant. Tyrosinase and MART-1 were also substituted with the combination of tyrosinase and MART-1 treated as one covariate (negative *versus* at least one positive). In the univariate analysis, a significant correlation was observed between a positive tyrosinase and MART-1 and a short relapse-free survival (HR 3.7, 95% CI, 1.5–9.1; $P=0.005$). Entering this covariate into the multivariate analysis together with gender, ulceration, thickness, Clark level, and histological subtype resulted again in thickness as the single independent prognostic factor, whereas the combination of tyrosinase and MART-1 was not significant (HR 1.5, 95% CI, 0.5–4.8; $P=0.11$). When analyzing tyrosinase and MART-1 results in only the baseline samples, no

Table 2. Frequency of patients with tyrosinase or MART-1 mRNA during follow-up after definitive surgery according to stage, relapse rate, and 5-year survival rate

AJCC stage	Number of patients (%)	Number of patients with tyrosinase or MART-1 mRNA (%)	Relapse rate *n* (%)	5-year overall survival (%)	Expected 5-year survival[1] (%)
IA	104 (44)	66 (63)	6 (6)	98	95
IB	59 (25)	31 (52)	6 (10)	90	90
IIA	32 (14)	23 (72)	8 (25)	66	78
IIB	28 (12)	18 (64)	13 (46)	64	65
IIC	13 (5)	4 (31)	9 (69)	54	45
Total	236 (100)	142 (60)	42 (18)	85	

AJCC, American Joint Committee on Cancer version 2001; MART-1, melanoma antigen recognized by T cells.
[1]Expected 5-year survival rate according to Balch *et al.* (2001).

Table 3. Univariate and multivariate Cox analyses of potential parameters of relapse-free survival in patients with AJCC stage I–II melanoma

		Univariate analyses	Multivariate analysis[1]		
Covariates	**Categories compared**	***P*-value**	**Hazard ratio**	**95% CI**	***P*-value**
Updated covariates					
Tyrosinase mRNA	Negative *versus* positive	0.03	2.7	0.4–16.7	0.36
MART-1 mRNA	Negative *versus* positive	0.06	3.1	0.9–10.2	0.07
Fixed covariates					
Gender	Female *versus* male	0.001			0.19
Age	< median *versus* ⩾median	0.13			
Ulceration	None *versus* ulceration	<0.0001			0.14
Primary tumor site	Limbs *versus* others	0.15			
Thickness (log transformed)	Continuous covariate	<0.0001	4.1	2.7–6.2	<0.0001
Clark level	I–III *versus* IV–V	<0.0001			0.22
Histological subtype	SSM *versus* others	<0.0001			0.75

AJCC, American Joint Committee on Cancer version 2001; MART-1, melanoma antigen recognized by T cells.
[1]All other significant covariates from the univariate analyses were not significant and therefore excluded from the model. *N*=224 patients, 38 relapses.

significant correlation to relapse-free survival was observed (data not shown).

DISCUSSION

Studies in which circulating tumor cells are detected with RT-PCR technology in serial blood samples are rare as demonstrated in a recent meta-analysis (Quaglino *et al.*, 2004). We identified five studies comparable to our own as shown in Table 4. Only patients with stage I–II melanomas were included in this table, but the total number of patients in these studies is noted. Disease stage in these studies was reported according to the AJCC version 1994, and our data were adjusted accordingly. Two studies included patients with stage II–III disease (Garbe *et al.*, 2003; Voit *et al.*, 2005), whereas the other studies included patients with stage I–IV disease (Curry *et al.*, 1999; Hanekom *et al.*, 1999; Brownbridge *et al.*, 2001). Curry *et al.* (1999) showed tyrosinase to be an independent prognostic factor for disease-free survival in the subgroup of patients with disseminated relapse, whereas MART-1 was an independent prognostic factor for disease-free survival in the subgroup of patients with loco-regional relapse. However, the analyses were performed in subgroups and not in the total patient cohort. The recent study by Voit *et al.* (2005) indicated a strong association between tyrosinase and a reduced disease-specific survival. The conclusion in these two studies was primarily based on patients with stage II–III disease. The three other studies did not report a prognostic impact of tyrosinase and/or MART-1, although the follow-up time in the study by Garbe *et al.* is too short for any valid conclusion.

There are a number of differences between our study and the above referenced. Only the study by Hanekom *et al.*

collected blood samples from the time of diagnosis as we did. Apart from our own study, only Voit *et al.* analyzed tyrosinase as an updated covariate in a Cox regression analysis. This method can evaluate serial measurements in the context of time and should be considered in studies analyzing serial measurements. In our study, the presence of tyrosinase and MART-1 mRNA in serial blood samples from the time of diagnosis and during the follow-up time was not independent prognostic factors for relapse-free survival, but MART-1 ($P=0.07$) approached the 5% significance level. In a recent meta-analysis specifically addressing the relationship between serial blood samples during follow-up in relation to relapse, it was concluded that tyrosinase mRNA was a potentially useful marker, at least in the follow-up of stage III patients (Curry *et al.*, 1999; Osella-Abate *et al.*, 2003; Quaglino *et al.*, 2004). In our study, 12% of stage I patients were positive for tyrosinase or MART-1 in the baseline sample compared to 13% of patients with stage II. Additionally, no difference in the frequency of positive samples was seen between stages I and II during the follow-up period (Table 2). This is in contrast to a number of studies in which the frequency of a positive sample increases significantly with stage (Mellado *et al.*, 1996; Palmieri *et al.*, 1999; Proebstle *et al.*, 2000).

None of the 89 control individuals in our study were positive for tyrosinase, but two individuals (2%) with benign nevi had one sample positive for MART-1. In the literature, Keilholz *et al.* (2004) has previously reported false-positive MART-1 analyses in three of 21 healthy volunteers. None of the two positive controls in our study were later diagnosed with a relapse or a new melanoma during the follow-up periods of 4.5 and 8 years, respectively. It remains an academic discussion whether these two samples were false positive or that these patients had a non-diagnosed melanoma. The latter option cannot be ruled out because all control patients with nevi were included in the study with the clinical suspicion of a melanoma. Reports of positive tyrosinase determinations in control subjects are rare. The meta-analysis by Tsao *et al.* (2001) suggested that the false-positive rate is about 0.37%. However, the false-positive rate is probably underestimated, as none of the studies, including our own one, examined serial control samples. Thus, the percentage of positive findings in our controls may have been higher, if several samples would have been taken. One explanation of false-positive samples could be contamination during the RNA extraction and RT-PCR analysis. However, RNA extraction, PCR reagent setup, and handling of PCR products were performed in separate rooms to avoid potential cross-contamination and negative and positive controls were used with each and every RT-PCR analysis.

We compared the 5-year survival rates in our patients with the material published by Balch *et al.* (2001). There was a good correlation stage by stage, especially in stage I patients, indicating that the patients in our material acted as expected.

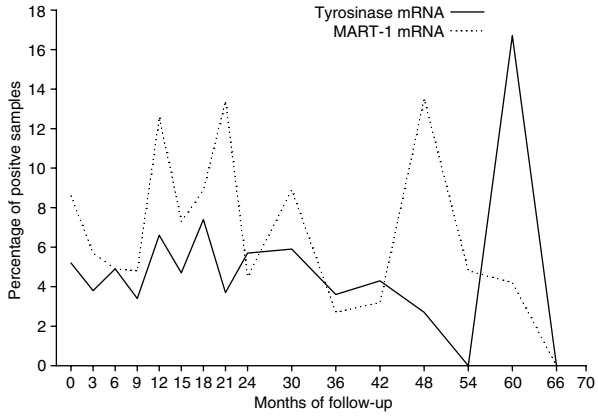

Figure 1. The percentage of tyrosinase and MART-1 mRNA-positive blood samples from 236 patients with AJCC stage I–II melanoma at the time of diagnosis (month 0) and during follow-up.

Table 4. Studies on consecutive RT-PCR detection of tyrosinase and MART-1 mRNA in AJCC stage I–II melanoma

			AJCC stage						
Study (year)	**Marker(s)**	**Total number**	**I**	**II**	**Samples collected from**	**Median number of assays per patient**	**Median follow-up (months)**	**Multivariate analysis**	**Interpretation**
Curry *et al.* (1999)	Tyrosinase, MART-1	186	13	76	Definitive surgery+ follow-up	NR	35	Yes	Positive[1]
Hanekom *et al.* (1999)	Tyrosinase	143	76	67	Definitive surgery	NR	48	NR	Negative[2]
Brownbridge *et al.* (2001)	Tyrosinase, MART-1	299	28	41	Definitive surgery+ follow-up	3	NR	NR	Negative[2]
Garbe *et al.* (2003)	Tyrosinase, MART-1	296	0	167	Follow-up	NR	19	NR	Negative[3]
Voit *et al.* (2005)	Tyrosinase	111	0	78	Follow-up	NR	75	Yes	Positive[3]
This study (2006)	Tyrosinase, MART-1	236	157	79	Definitive surgery	10	66	Yes	Negative

AJCC stage, American Joint Committee on Cancer stage version 1994; MART-1, melanoma antigen recognized by T cells; NR, not reported; n, total number of patients with stage I and II disease.
[1]Conclusion was based on data from stage I–III disease.
[2]Conclusion was based on data from stage I–IV disease.
[3]Conclusion was based on data from stage II–III disease.

Patients with stage IIc had a better 5-year survival than expected and patients with stage IIa had a worse survival. This was probably owing to the low number of patients in each of stages IIa–c and the fact that 12 patients with missing tumor thickness were assigned to stages IIb and c.

Our data showed that tyrosinase and MART-1 was infrequently detected (5–7% of the total samples). However, based on the high number of serial samples, we observed a high frequency of patients with at least one positive sample as seen in Table 2. In fact, tyrosinase or MART-1 was detected during follow-up in 60 and 62% of patients with stage I–II disease, respectively. Our data raises the question whether tyrosinase and/or MART-1 mRNA in peripheral blood as an indicator of circulating melanoma cells have any clinical implications for the patient. A recent study in breast cancer patients detected circulating tumor cells in 13 of 36 patients between 7 and 22 years after mastectomy in patients without the evidence of malignant disease (Meng *et al.*, 2004). The half-life of circulating tumor cells in breast cancer was reported between 1 and 2.4 hours! The authors concluded that these patients must have had tumor cells somewhere in the tissues, that replenished the dying cells in the blood, and that this could represent one of the mechanisms underlying tumor dormancy. Dormancy and long-term relapses are also well known in melanoma (Shaw *et al.*, 1985; Tsao *et al.*, 1997), but the reported frequency of long-term relapses is reported as low as 0.7% (Tsao *et al.*, 1997). The low ''false-positive'' rate in controls in our study suggests that stage I–II melanoma patients with a positive RT-PCR result may have dormant tumor cell deposits. Thus, in order to examine whether melanoma patients continue to have intermittent positive samples without relapsing, a re-analysis after 10–15 years is warranted. However, the frequency of patients being at least once positive was much greater than the expected relapse rate and deaths in stage I–II melanoma (Table 2). It is not realistic, given the length of our follow-up that many more patients will relapse.

In conclusion, our study cannot demonstrate that tyrosinase and MART-1 mRNA has independent prognostic impact on relapse-free survival in stage I–II melanoma patients.

MATERIALS AND METHODS

Patients

The study was conducted as a prospective collection of blood samples in 236 consecutive patients with AJCC stage I–II melanoma from February 1997 to December 2002. The local ethics committee of Aarhus County accepted the present project, and a written informed consent was obtained from each patient. The study was conducted according to the Declaration of Helsinki Principles. Blood samples were collected before definitive primary surgery and during follow-up until 2 years after inclusion of the last patient.

Baseline staging procedures for all patients included a chest X-ray together with a full blood count including liver enzymes. Sentinel node procedures were not performed routinely during this study. Disease status was coded according to the AJCC guidelines version 2001 (Balch *et al.*, 2001). Tumor thickness could not be measured in 12 patients (5%) owing to incomplete resection (eight patients), regression (one patient), and technical problems (three patients), respectively. These patients were coded as thick melanomas (stage IIB), except for lesions with ulceration, which were coded as IIC. Follow-up was performed every 3 months for the first 2 years and then every 6 months up to 5 years. At each visit, a clinical history, physical examination, and blood samples for RT-PCR analyses were obtained. Diagnostic imaging, that is, X-ray, ultrasonography, or computed tomography were performed if clinically indicated. In the event of regional or distant relapse, patients were excluded from this study; only patients with a local relapse were followed after surgical excision.

Control samples were obtained from 89 individuals, 35 had benign nevi and 55 had a cancer other than melanoma. The latter group consisted of a variety of cancers, such as gynecological ($n=11$), breast ($n=10$), urogenital ($n=7$), head and neck ($n=7$), skin ($n=6$), sarcoma ($n=5$), lung ($n=4$), and others ($n=6$).

Reverse transcriptase-PCR

To avoid contamination by normal skin melanocytes during blood sampling, the blood for RNA extraction were collected after serum samples (Hanekom *et al.*, 1997). The RNA extraction, PCR reagent setup, and handling of PCR products were performed in separate rooms to avoid potential cross-contamination in PCR. Total cellular RNA was extracted using the RNeasy blood mini kit (Qiagen, Chatsworth, CA) as outlined by the manufacturer. All samples were processed within 4 hours on the day of collection. Samples were stored at $-80^{\circ}C$ until analyzed in a blinded manner. Primer sequences and RT-PCR conditions have been described previously (Schmidt *et al.*, 2002). Quantification of the patient RNA samples was performed with a calibrated RT-PCR design as described previously (Sorensen *et al.*, 2000). Integrity of RNA was determined by performing parallel RT-PCR assays using primers specific for the housekeeping gene β-actin. Blood samples that failed to amplify products for β-actin RNA were considered non-informative and discarded.

Statistics

The Wilcoxon rank-sum test and Spearman correlation were used to test for associations and correlations between baseline tyrosinase and MART-1 mRNA on one hand and clinical and pathological characteristics on the other hand. Relapse-free survival was calculated as the time from definitive surgery to the date of progression or last follow-up. For relapse-free survival, patients without an updated measurement (approximately 6 months) after the latest update were removed from the risk set and only re-entered if a measurement subsequently was available. All data on duration of disease-free survival were updated on 1 May 2005 and calculations were carried out using SAS software (SAS Institute, Cary, NC; version 9.1). All *P*-values are two-sided and values less than 5% are considered significant.

The simultaneous relationship of multiple prognostic factors for survival was assessed using Cox's proportional-hazards model with backward reduction. Factors with a *P*-value less than 0.10 in the univariate analyses were included in the multivariate analysis to identify factors of independent significance. The assumption of proportional hazards was verified graphically with log ($-$log (S)) *versus* time plots. The prognostic factors used for covariate selection were the following: gender (female *versus* male), age ($<$median age *versus* $\geqslant$median age), primary tumor site (limbs

versus other sites), ulceration (none *versus* ulceration), Clark level (level I–III *versus* IV–V), tumor thickness (logarithmically transformed values), and histological subtype (superficial spreading melanoma *versus* others). Tyrosinase/MART-1 mRNA (negative *versus* positive) was considered as updated (time-dependent) covariates because results changed over time, and several samples were available from each patient.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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