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Molecular Markers in Malignant Cutaneous Melanoma: Gift Horse or One-Trick Pony?

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Abstract The management of malignant cutaneous melanoma is problematic. Current clinical prognostic factors do not adequately predict disease recurrence and overall survival in a significant subset of patients. Adjuvant therapies for melanoma are notoriously toxic and associated with significant morbidity. Furthermore, it has been difficult to predict which patients will respond best to these treatments, if at all. DNA and RNA biomarkers have been developed to help overcome these problems. Biomarkers have been shown to upstage patients with melanoma, but are the assays sensitive and specific enough for clinical use as predictors of disease outcome or treatment response? We review our experience with DNA and RNA biomarkers in terms of their prognostic and predictive capabilities in malignant melanoma and outline their likely role in the future of melanoma staging, surveillance, and treatment. J. Cell. Biochem. 96: 473–483, 2005.

Key words: RT-PCR; qRT; DNA; RNA; molecular markers; melanoma

BACKGROUND

Adjuvant therapies have had limited or no impact on overall survival in melanoma. When possible, surgical resection is the preferred treatment for both early and advanced stages of disease. The treatment of localized melanoma (stage I/II) is straightforward and associated with an average 10-year survival rate of 85% [Balch et al., 2004]. The management of regional nodal (stage III) and distant metastatic disease (stage IV), however, remains the most challenging aspect of melanoma patient management.

Lymph node (LN) metastasis is the most important prognostic factor for 10-year survival. Patients with regional nodal metastasis experience a drastic drop in 10-year survival rates compared to patients without LN involvement. Although a heterogeneous group, stage III melanoma patients have an average 10-year survival rate of 35% [Balch et al., 2004]. LN dissection is advocated for clinically palpable nodes, but elective dissection of non-palpable LN is associated with significant morbidity and no clear benefit for the majority of patients. Lymphatic mapping and selective lymphadenectomy has facilitated the detection of subclinical regional nodal metastasis and avoids the morbidity of a complete LN dissection [Morton et al., 1992]. The technique is not perfect, however, up to 13% of patients with histopathologically negative sentinel lymph nodes (SLNs) will develop regional or distant disease recurrence, presumably due to a failure to locate the true SLN or identify metastatic deposits when the node is examined by hematoxylin and eosin (H&E) staining or immunohistochemistry (IHC) [Gershenwald et al., 1998]. The SLN technique accurately identifies subclinical nodal metastasis, but more sensitive techniques of detecting occult disease may better predict patients at risk of developing regional or distant metastases.

Distant metastases are more difficult. Patients with AJCC stage IV melanoma have a 10-year survival rate less than 10% [Balch et al., 2004]. The main reasons for this are twofold. First, adjuvant therapy for metastatic melanoma is

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grossly inadequate. Perhaps worse, such treatments are notoriously toxic and associated with significant morbidity. Better patient selection and stratification could increase response rates by improving the selection of candidates for adjuvant treatment. Patients unlikely to respond could avoid unnecessary and non-beneficial therapy. Second, metastatic disease is rarely detected early enough for adjuvant therapy to be effective. Advances in computed tomography (CT) scanning, magnetic resonance imaging (MRI), and fluorodeoxyglucose positron emission tomography (FDG-PET) scanning have allowed for earlier diagnosis of metastatic disease, but this has not translated into a benefit in overall survival.

Multiple RNA and DNA markers have been developed over the last decade as indicators of tumorigenesis, disease progression, recurrence, response to therapy, or predictors of survival. Molecular markers may represent tumor-associated antigens, tumor suppressor genes, oncogenes, transcription factors, or cellular apoptotic mediators. Reproducible, cost-effective and high-throughput assays may be applied to fluids or tissues that are easily sampled with little or no associated morbidity. The ability of these molecular techniques to upstage melanoma is without question and has been validated by several independent laboratories. Upstaging of disease has no clinical utility, however, unless it can be correlated with disease recurrence, progression, or overall survival. Can molecular markers improve outcome prediction and prognosis in patients with malignant melanoma? Is molecular upstaging too good to be true? A gift horse, or a one-trick pony?

Our laboratory has significant experience in the development of genomic DNA and RNA molecular markers for the detection of disease, and prediction of response to therapy and overall survival. While these molecular approaches may be applied to virtually any tissue, including cerebral spinal fluid and bone marrow, the most clinically relevant assays use specimens of peripheral blood and regional LNs.

MOLECULAR ANALYSIS OF TISSUE SPECIMENS

Occult disease in the SLN frequently represents the earliest stage of metastatic disease. It is established that the SLN represents the status of the draining lymphatic basin. The SLN status is important, not only for the prognostic staging information it provides, but for guiding treatment as well. Patients with SLN metastases should undergo a complete LN dissection and be offered adjuvant therapy; adjuvant treatments that are relatively ineffective in the setting of gross metastatic disease may be more effective against micrometastatic disease. If the pathological analysis of the SLN is falsely negative, however, a significant benefit of the procedure is lost.

Standard histopathologic analysis of the SLN incorporates both H&E staining and IHC, usually using antibodies to S100, HMB45, or MART-1 (MelanA). The node is typically bivalved and several additional sections may be examined. Despite this, routine pathological analysis of the SLN is prone to sampling error; even with step sectioning less than 5% of the node is sampled [Yu et al., 1999].

mRNA markers can be used to detect micrometastatic disease in SLNs that are negative for tumor by H&E and IHC, and molecular staging of the SLN has been correlated with disease-free status and overall survival. The upstaging of SLN using RT-PCR has been used for over a decade and incorporates single or multiple mRNA markers.

Multi-Marker RT-PCR

All cells within a tumor will not necessarily express a single marker. With RT-PCR, this is a binary event: either expression is present or absent. In early studies, gel electrophoresis was used to assess results of the single-marker RT-PCR assay. Such assays are inherently subjective and may fail to detect markers that are expressed in low copy numbers.

To increase the specificity of RT-PCR assay, investigators have utilized a multi-marker panel for SLN analysis. The chance that a tumor cell expresses one of four markers greatly increases the likelihood of detection. In addition, the expression of more than one marker decreases the risk of false-positive findings due to expression of a marker on non-melanoma cells. Investigators have documented high false-positive rates using tyrosinase as a single marker [Prichard et al., 2003]. Possible reasons include detection of illegitimate transcription, benign nevi cells, or PCR contamination. Multiple molecular markers improve assay sensitivity and specificity by addressing tumor cell heterogeneity, mRNA half-life, and variations in mRNA quantity and quality.

Bostick et al. [1999] used a panel of tyrosinase, melanoma antigen gene-3 (MAGE-3), and melanoma antigen recognized by T-cells-1 (MART-1) to detect micrometastases in the SLNs of 72 stage I-III melanoma patients. MAGE-3 is part of the *MAGE* gene family and is expressed in male germline cells and in tumors of various histologies. MAGE-3 codes for a tumor-associated antigen that can be recognized by T-lymphocytes. MART-1 is a melanoma-associated antigen recognized by HLA-A2-restricted tumor-infiltrating lymphocytes. The RT-PCR assay was considered positive if $\geq$2 markers were detected. When results were compared to standard H&E plus IHC, RT-PCR markers were positive in 94% of histologically positive SLN metastases and 36% of histopathologically negative SLNs. After 12 months of follow-up, RT-PCR positive patients had a significantly higher rate of disease recurrence. RT-PCR was a better predictor of recurrence-free survival than H&E plus IHC. In addition to upstaging the regional nodes in a significant proportion of patients with histopathologically negative SLN, the multi-marker RT-PCR predicted a survival disadvantage in patients with RT-PCR positive SLNs.

FROZEN VERSUS FIXED SPECIMENS

To minimize sampling error, investigators at the John Wayne Cancer Institute (JWCI) developed a technique for parallel preparation of frozen SLN tissue for histopathologic and molecular analysis: the SLN is bivalved and each half is sequentially sectioned. Frozen sections are obtained from each half of the node and submitted for molecular studies. The remainder of each half is then fixed in formalin and embedded in paraffin. Wth each half embedded in the same paraffin block, diagnostic slides are cut in parallel to the slides submitted for molecular studies. This provides a more uniform molecular assessment of the SLN relative to the portion submitted for histopathological diagnosis. However, even with careful sectioning techniques, the use of frozen specimens remains suboptimal; reliance on frozen specimens may sacrifice tissue necessary for an accurate pathological diagnosis and the acquisition and storage of frozen tissues may be prohibitively expensive and labor-intensive.

Kuo et al. [2003] reported the analysis of paraffin-embedded (PE) SLNs by a semi-quantitative RT-PCR and electrochemiluminescence (ECL) assay. Unlike frozen tissue, PE tissues are readily available after a pathologic diagnosis is established and require no special storage conditions or processing. PE tissues have the added advantage of resisting mRNA degradation during storage and therefore may be archived and used for retrospective analyses. In this study, tyrosinase-related protein-1 (TRP-1), tyrosinase-related protein-2 (TRP-2), tyrosinase, and MART-1 were used as molecular markers. TRP-1 and -2 are melanosomal membrane glycoproteins recognized by T-cells. At least one RT-PCR marker was positive in 95% of the 37 patients with metastases identified by H&E or IHC. The number of positive markers correlated significantly with the primary melanoma's Breslow thickness, an established clinical prognostic factor. In the 40 histopathologically negative SLNs, RT-PCR expression of at least two markers significantly correlated with disease recurrence and overall survival after a median follow-up of 55 months.

Quantitative Real-Time PCR

Semi-quantitative ECL assays foreshadowed the development of quantitative real time PCR (qRT). With the advent of qRT, better control of results was obtained. qRT provides a means of quantifying individual marker copy numbers rather than relying on subjective Southern blot assays that, at best, can only distinguish the presence or absence of a given marker. qRT is particularly valuable when detecting occult tumor cells, as this assay better distinguishes differential biomarker expression among tumors, even at low copy numbers.

Takeuchi et al. [2004] performed a qRT assay on 53 histologically-confirmed positive and 162 negative PE SLN using a multi-marker panel of MART-1, MAGE-3, $\beta 1 > 4$-N-acetylgalactosaminyl-transferase (GalNac-T), and paired-box homeotic gene transcription factor 3 (Pax3). GalNac-T is a key enzyme in the synthesis of cell-surface gangliosides GM2 and GD2 that are abundant in melanoma cells. Pax3 is a transcription factor that participates in melanocyte development. Patients with $\geq$1 positive marker had significantly higher rates of disease recurrence at a median follow-up of 60 months. Furthermore, those with 1, 2, and 3 positive qRT markers had proportional and statistically significant decreases in disease-free survival. Patients with $\geq$1 positive marker had

significantly worse overall survival. An increasing number of positive markers correlated with a decrease in disease-free and overall survival.

The ability to detect molecular marker expression in SLNs has tremendous clinical significance. Molecular marker expression in SLNs can be used to stratify patients into categories of risk for both disease-free and overall survival. Patients with positive SLNs by conventional and qRT assessment experience the worst outcomes, whereas those with negative SLNs using these combined techniques have the best prognosis. Interestingly, the risk associated with qRT-positive SLNs is not uniform; it seems to vary directly with the number of molecular markers detected [Takeuchi et al., 2004].

Prospective clinical trials are underway to validate panels of molecular markers as a basis for risk stratification: patients at highest risk may benefit from more rigorous clinical follow-up, radiographic surveillance, and adjuvant treatment, while those at lowest risk may be followed less aggressively and avoid unnecessary adjuvant treatment. Of particular interest is the second multicenter selective lymphadenectomy trial (MSLT-II), a phase III international trial and the first major melanoma clinical study to randomize patients according to qRT assessment of the SLN. MSLT-II will accrue 3,500 patients from more than 30 countries. SLNs that are tumor-negative by H&E and IHC will be examined by multi-marker qRT at JWCI. Patients whose qRT results are negative will undergo routine follow-up, whereas those with positive qRT results will be randomly assigned to one of two treatment arms: observation or complete LN dissection. If qRT evidence of LN metastasis represents clinically significant occult disease, then patients who undergo nodal dissection should have a significantly better survival than those assigned to observation.

We have also used a multi-marker qRT assay to predict outcome in patients with distant melanoma metastases (AJCC stage IV). Takeuchi et al. [2003] used the immunogenic melanoma differentiation antigens tyrosinase, TRP-2, and MART-1 as qRT markers and demonstrated that loss of these markers was associated with poorer overall survival (Fig. 1). Loss of immunogenic melanoma-associated antigens may aid tumor cells in avoiding detection and elimination by host antigen-specific immunity, thereby facilitating tumor progression.

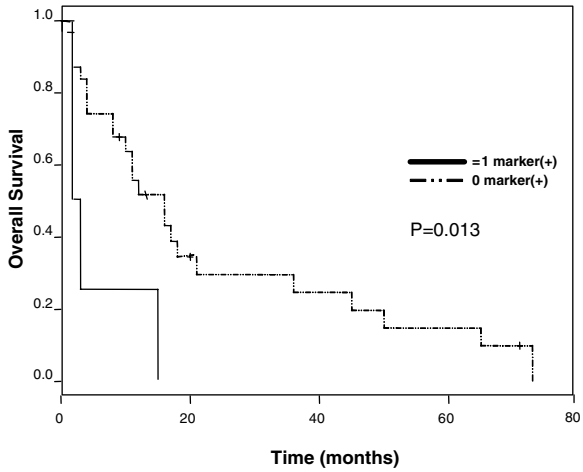

Fig. 1. AJCC stage IV melanoma patients expressing 1 immunogenic melanoma associated antigen demonstrate improved overall survival following surgical resection compared to patients that do not express such antigens assayed by qRT.

DNA Markers

The analysis of DNA for somatic mutations, LOH or tumor-related gene methylation has several advantages compared to mRNA-based assays in melanoma. DNA is more stable than RNA, so assays are less technically challenging to perform. While mRNA expression may be variable and difficult to quantify objectively using standard RT-PCR assays, DNA markers detect a wide variety of genetic anomalies. DNA markers for somatic alterations of tumor-related genes and oncogenes are tumor-specific and not as vulnerable to false-positive results as mRNA assays.

ALLELIC IMBALANCES

Various malignancies have demonstrated allelic imbalances (AI). Loss of heterozygosity (LOH) is not typical in non-tumor tissues. Its presence suggests the alteration of genomic regions related to cellular regulatory genes, tumor suppressor genes, or oncogenes that can influence oncogenesis or tumor progression. A combination of genetic linkage and LOH analysis in patients with familial melanoma and dysplastic nevi syndrome led to the discovery of several informative loci [Florenes et al., 1994; Isshiki et al., 1994; Celebi et al., 2000]. LOH of microsatellites, highly repetitive, polymorphic sequences of base pairs, represents a common genetic alteration in melanoma and occurs on multiple chromosomes [Healy et al.,

1995]. Increasing levels of AI have been correlated with a poorer disease outcome in various cancers, including melanoma [Mao et al., 1994].

Fujimoto et al. [2004b] used four microsatellite markers surrounding the APAF-1 locus to detect LOH in 44 patients with regional nodal metastases and 39 with in-transit metastases. APAF-1 is a candidate tumor suppressor gene at chromosomal region 12q22-23 that coordinates the mitochondrial apoptotic pathway downstream from p53. AI of APAF-1 may facilitate oncogenic transformation, tumor growth, and disease progression. Regional nodal metastases expressed LOH of $\geq$1 marker in 28% of cases, a rate significantly higher than that seen in primary tumors of equivalent stage, suggesting that APAF-1 may play a key role in melanoma progression. In addition, LOH of $\geq$1 marker in LN metastases correlated with decreased overall survival after 27 months of follow-up (Fig. 2).

MUTATIONS

Oncogene and tumor suppressor gene mutations are rare in melanoma. Deletions and mutations of known tumor suppressor genes such as *N-Ras*, *P53*, and *CDKN2* have been reported, but occur at a low frequency and likely play a limited role in sporadic cutaneous melanoma [Florenes et al., 1994; Holland et al., 1994; Hussussian et al., 1994; Isshiki et al., 1994; Essner et al., 1998; Celebi et al., 2000]. Few mutations occur with a frequency greater than 25% in melanoma. B-type Raf kinase oncogene (BRAF), one of three Raf proteins in vertebrates, is an exception to this rule. BRAF encodes a key serine/threonine kinase important in the mitogen-activated protein kinase pathway (MAPK) for the transduction of signals from the Ras oncogene. Mutations of BRAF significantly increase kinase activity and promote transcription-mediated proliferation and neoplastic growth. Up to 92% of BRAF mutations in melanoma are due to a 1796 T $\rightarrow$ A nucleotide transversion that results in a mutant V600E amino acid. Missense BRAF mutations have been reported in six of nine (66%) primary melanomas [Davies et al., 2002] and a subsequent study demonstrated nearly identical mutation rates in nevi, primary melanoma and metastatic melanoma [Pollock et al., 2003]. Debate is ongoing as to whether BRAF mutations correlate with melanoma development and progression or are merely a bystander genetic event. Although other studies have reported nearly equivalent rates of BRAF mutation occurring in nevi, primary tumors and metastatic tumors, favoring a minimal role for BRAF in tumor progression, our data do not support this conclusion, particularly in later stages of disease.

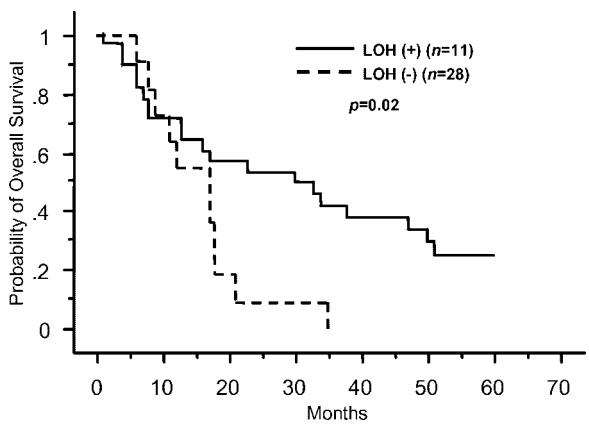

Fig. 2. AJCC stage III melanoma patients with LOH of $\geq$1 microsatellite marker at the APAF-1 locus (12q22-23) in LN metastases demonstrate decreased overall survival after 27 months of follow-up.

Shinozaki et al. [2004] examined 68 metastatic tumors for mutations of BRAF using PCR and automated capillary array electrophoresis (CAE). Twenty of the tumors examined involved regional LN. When compared to BRAF mutations in primary melanomas, the rate in regional nodal metastases was significantly higher (30% vs. 50%).

EPIGENETICS: HYPERMETHYLATION

The role of gene promoter region CpG island hypermethylation in cancer development has become a thriving area of investigation. Genes may be transcriptionally silenced when their promoter region CpG islands contain methylated cytosines 5′ to an adjacent guanine. Promoter region hypermethylation of cancer-related genes can be as functionally significant as genetic mutations or deletions in permitting neoplasia and facilitating tumor progression [Jones and Baylin, 2002]. The hypermethylation of gene promoter regions is a powerful mechanism of silencing gene expression. Perhaps more so than other genetic aberrations, hypermethylation of tumor-related gene promoter regions may play a prominent role in melanoma development and progression. The detection of this epigenetic event can be used

as a predictive marker of disease outcome. Spugnardi et al. [2003] detected hypermethylation of the candidate tumor suppressor gene, RAS association domain family protein 1A (RASSF1A) in 3/9 (33%) regional metastases by MSP. Another study quantified RASSF1A and retinoic acid receptor β-2 (RAR-β2) promoter region hypermethylation in 37 melanoma patients with clinically positive LNs [Yao et al., 2004]. Hypermethylation was detected for RASSF1A alone in 16%, RAR-β2 alone in 28%, and both in 14%. By multivariate analysis, RAR-β2 hypermethylation correlated with decreased disease-free and overall survival. Hoon et al. [2004] examined the promoter region hypermethylation of four candidate tumor suppressor genes (RAR-β, RASSF1A, MGMT, and DAPK) via MSP in 86 metastatic melanoma tumors. The gene methylation frequencies were are presented in Table I. Tumor-related gene hypermethylation is more common in metastatic compared to primary tumors; this indicates a possible role of this epigenetic event in tumor progression. Larger patient populations and additional genes need to be analyzed to further assess the role of epigenetic changes as prognostic factors in advanced metastatic melanoma.

MOLECULAR ANALYSIS OF BLOOD SPECIMENS

Most tumors metastasize and disseminate hematogenously, including melanoma. The detection of tumor-associated molecular markers in blood is therefore a logical, accessible, and convenient alternative to the examination of primary or metastatic tumors. Routine blood draws are relatively non-invasive, which makes it possible to monitor molecular markers of disease progression, recurrence or response to therapy at multiple time-points with little associated morbidity.

Multi-Marker RT-PCR

Several authors maintain that the detection of tyrosinase in blood-based RT-PCR assays has no clinical utility in melanoma [Foss et al., 1995; Glaser et al., 1997; Reinhold et al., 1997; Farthmann et al., 1998; O'Connell et al., 1998]. In stage III and IV disease, when tumor burden is presumably greatest, the detection of tyrosinase mRNA has been highly variable, ranging from 0% to 100% [Brossart et al., 1993; Battayani et al., 1995]. Differences in methods of RNA purification and extraction, PCR set-up and cycling, as well as differences in data interpretation may be responsible for these disparate findings. Tumor biology may influence results as well. Tumor heterogeneity may lead to clones of cells that do not express the marker, and the presence of normal cellular transcripts detected by PCR may dilute the tumor-related mRNA. Daily fluctuations in tumor cell shedding will also contribute to variation in the detection of tyrosinase mRNA between patients, particularly those that have received different treatment. Thus the use of tyrosinase as a single marker of melanoma progression or disease recurrence and predictor of overall survival in LN metastases seems an inadequate, one-dimensional assay for a process that is decidedly complex and multi-dimensional.

TABLE I. Hypermethylated Genes in 86 Metastatic Melanoma Tumors

	RAR-β2	RASSF1A	MGMT	DAPK
Promoter region methylation rate (%)	70	57	34	19

Investigators have improved the sensitivity and specificity of the RT-PCR assay by employing multi-marker panels in peripheral blood analysis [Hoon et al., 1995; Curry et al., 1998; Bostick et al., 1999; Hoon et al., 2000; Boi et al., 2002; Kuo et al., 2003; Takeuchi et al., 2004]. Hoon et al. [1995] used a multi-marker panel of tyrosinase and tumor-associated antigens melanotransferrin (p97), melanoma antigen gene A3 (MAGE-3) and an adhesion-related glycoprotein, melanoma cell adhesion molecule (MUC18) to assay the blood of 119 stage I–IV melanoma patients by RT-PCR. The four markers assay was superior to the tyrosinase assay and correlated with both disease stage and progression. An identical multi-marker panel was used by the same group in blood specimens from 46 patients with AJCC stages II, III, or IV melanoma, who were followed up for $\geq$4 years. The number of positive RT-PCR markers approached significance as a predictor of overall survival ($P = 0.068$) [Hoon et al., 2000]. The number of positive markers used alone or in combination with AJCC stage was a significant predictor of disease recurrence ($P = 0.024$ and $P = 0.037$, respectively). Studies based on larger numbers of patients will better define the role of MUC18 and p97 in melanoma development

and progression. Furthermore, these markers should be validated in other tissue types.

Wascher et al. [2003] used a panel of tyrosinase, melanoma antigen recognized by T-cells-1 (MART-1), and universal melanoma antigen gene-A (uMAGE-A). The primers for uMAGE-A were designed to detect ≥6 members of the MAGE-A gene family. Semi-quantitative ECL confirmed all RT-PCR products. Thirty stage III patients matched for known clinical prognostic factors and rendered disease-free by surgical resection prior to treatment with a melanoma vaccine were assayed in this retrospective study. Peripheral blood was obtained before the first vaccination, and prior to vaccinations at 8 and 16 weeks. This study sought to use the multi-marker RT-PCR assay to detect occult circulating tumor cells early in the course of adjuvant therapy. Currently, no validated blood marker for melanoma has reliably predicted response to adjuvant therapy. A correlation between circulating tumor cells detected by RT-PCR and disease recurrence or decreased overall survival would be extremely beneficial to the clinician. In this study, blood samples were obtained during the course of adjuvant therapy, so tumor cells detected by RT-PCR could be correlated with disease recurrence and overall survival at multiple time-points. Median follow-up in this study was 74 months. Of those who experienced disease recurrence, 53% had ≥1 RT-PCR marker positive. The presence of ≥1 positive RT-PCR marker was associated with an increased risk of disease recurrence in multivariate analysis (Fig. 3). Similarly, of patients dying during the follow-up period, 53% had ≥1 RT-PCR marker positive in serial peripheral blood specimens. The presence of ≥1 positive RT-PCR marker was significantly associated with decreased overall survival (Fig. 4). This study demonstrated that blood-based multi-marker RT-PCR assays have clinical utility in predicting disease recurrence, overall survival, and response to adjuvant therapy.

Others have attempted to correlate biomarker expression with various treatment modalities, but have failed to demonstrate a correlation with risk of disease recurrence or improvement in overall survival [Curry et al., 1998; Palmieri et al., 2001]. We recently developed a blood-based qRT assay incorporating markers initially used to upstage SLN [Takeuchi et al., 2004]. Serial blood draws assessed by multi-marker qRT successfully detected tumor

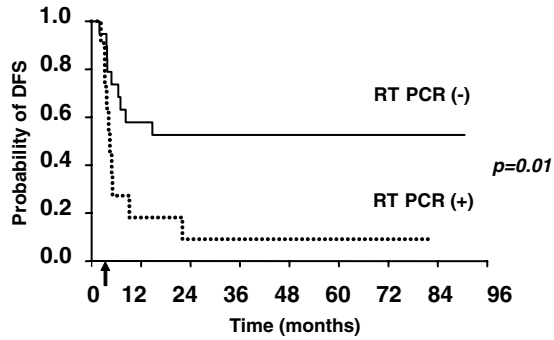

Fig. 3. RT-PCR results of 30 stage III rendered disease-free by surgical resection prior to treatment with a melanoma vaccine with median follow-up of 74 months. Patients with ≥1 marker detected by RT-PCR were considered RT-PCR (+). Of those who experienced disease recurrence, 53% had ≥1 RT-PCR marker positive (+).

cells in the blood of stage III melanoma patients receiving neoadjuvant biochemotherapy (BC) [Koyanagi et al., 2005]. Patients who demonstrate a decrease in circulating tumor cells during therapy tumor have significantly lower rates of disease recurrence compared to patients who show no change in the number of circulating tumor cells (Koyanagi, personal communication). We have shown that multi-marker qRT can predict treatment outcome and monitor the effectiveness of other modes of treatment.

DNA Tumor Markers

Free, circulating tumor-related DNA has been reported in the peripheral blood of patients with a variety of cancers, including melanoma.

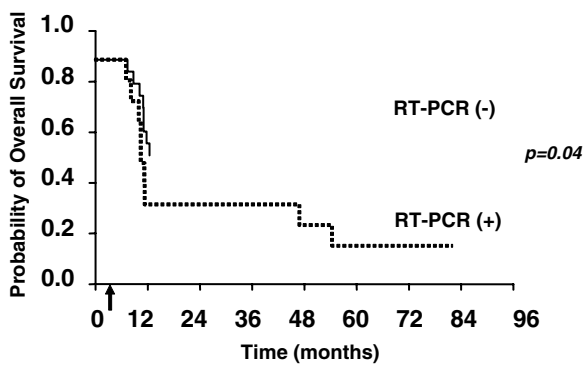

Fig. 4. RT-PCR results of 30 stage III rendered disease-free by surgical resection prior to treatment with a melanoma vaccine with median follow-up of 74 months. Of patients dying during the follow-up period, 53% had ≥1 RT-PCR marker positive (+) in serial peripheral blood specimens and this was significantly associated with decreased overall survival.

The origin of circulating tumor-related DNA may be necrotic, apoptotic, or physically disrupted tumor cells. Free circulating tumor-related nucleic acids have demonstrated diagnostic and prognostic importance and predictive relevance [Nakayama et al., 2000].

Free circulating tumor-related DNA may be detected as LOH of DNA microsatellites, methylated DNA, or mutations. Free circulating nucleic acids in peripheral blood demonstrate genetic aberrations similar to those found in the primary tumor [Fujiwara et al., 1999; Nakayama et al., 2000]. Circulating DNA may therefore have clinical utility as a marker of disease recurrence following surgery. Similarly, the detection of circulating tumor DNA during the administration of adjuvant therapy may be used as a predictor of therapeutic response.

Fujiwara et al. [1999] were the first to show a correlation between circulating DNA and disease progression in melanoma. The plasma of 76 stage I–IV melanoma patients was examined for microsatellite loss using ten markers (D1S214, D1S228, D3S1293, D6S264, IGFIIR, D9S157, D9S161, D10S212, D10S216, and D11S925) on six chromosomes. LOH of $\geq$1 marker was found in 50% of patients and correlated with disease stage.

Using a different panel of eight microsatellite markers (D1S214, D1S228, D3S1293, D6S264, D9S157, D9S161, D10S216, and D11S925) on six chromosomes, Taback et al. [2001] analyzed LOH in preoperative and postoperative blood specimens from 57 patients who underwent complete surgical resection of early or advanced melanoma. LOH of at least one marker occurred in 56% of patients and correlated with disease stage. In patients with metastatic melanoma (stage III/IV), LOH positivity correlated with a significant risk of death after a median follow-up of 21 months. If genetic instability in melanoma increases with advancing AJCC stage, then AI becomes a useful marker in patients with regional and distant metastases. For this reason, LOH has been evaluated as a predictor of response to therapy in patients with advanced-stage melanoma.

Fujimoto et al. [2004a] used a 4-marker panel of microsatellites surrounding the APAF-1 locus at 12q22-23 to detect LOH in the sera of 49 patients who received BC for stage IV melanoma. Patients were categorized as BC responders or non-responders. Non-responders had significantly more LOH than did responders; those with LOH of 12q had significantly worse overall survival. Taback et al. [2004] were the first to prospectively document the association between circulating DNA markers and a patient's response to adjuvant therapy for melanoma. They examined the blood of 41 stage IV melanoma patients receiving BC for LOH of 9 markers on 7 chromosomes. Patients were categorized as BC responders or non-responders. Responders demonstrated clinical and radiographic complete or partial responses while non-responders showed either stable or progressive disease. At the start of BC a significant difference in LOH was noted between responders and non-responders. Only 9% of responders exhibited LOH of $\geq$1 marker compared to 56% of responders. Patients with LOH of $\geq$1 marker had significantly decreased median progression-free and overall survival compared to patients without LOH (Fig. 5).

The utility of circulating tumor-related DNA detection lies in the ability to serially assay tumor markers without sampling either the primary or metastatic tumor, which may be unavailable or inaccessible. Serial determinations of biomarker status have the most significant implications in the context of adjuvant therapy administration. Molecular assays to

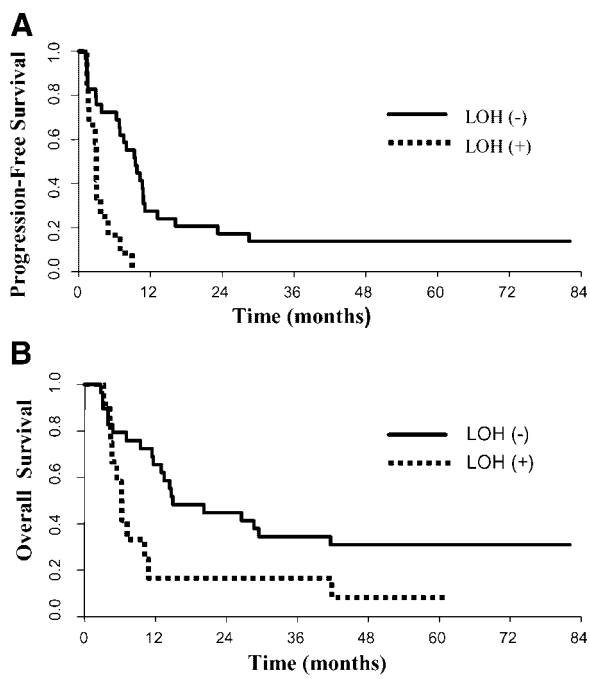

Fig. 5. Kaplan–Meier graph of progression-free (**A**) and overall (**B**) survival in 41 AJCC stage for melanoma patients according to serum LOH of 9 microsatellite markers prior to initiation of biochemotherapy.

accurately predict, which patients are most likely to respond would immeasurably improve selection of candidates for adjuvant therapy and allow early implementation of alterative strategies for those who are unlikely to respond.

CONCLUSIONS

Molecular markers will become invaluable prognostic indicators as clinicians seek to stratify patients for clinical trials, adjuvant treatment, and clinical follow-up regimens. The use of molecular markers to investigate clinical melanoma specimens will lead to new and more sensitive diagnostic evaluations and, potentially, much-needed treatments.

DNA and RNA molecular markers will assume an important role in the management of cutaneous melanoma. Assays based on these markers can be used to increase diagnosis of subclinical metastatic disease. Such molecular upstaging will in turn improve the stratification of patients for clinical trials. More importantly, though, molecular upstaging allows clinicians to better characterize and predict tumor behavior with respect to disease recurrence and likelihood of long-term survival. Biomarker-derived data regarding disease recurrence risk and overall survival will influence the selection of therapy and the frequency of follow-up. When an adjuvant treatment regimen is selected, biomarker assays to detect free circulating tumor-related DNA or circulating tumor cells can determine the likelihood of response; during treatment, these assays can serve as a surrogate marker of clinical response.

Our objective is to use a platform of molecular markers to detect clinically relevant occult tumor cells in peripheral blood and SLNs. This information will be used for ultrastaging of disease, a process that moves beyond standard clinical staging. The role of molecular biomarkers—and ultrastaging—in the management of melanoma can only be validated by data from multicenter randomized controlled trials such as MSLT-II, a trial that will determine the benefit of multi-marker qRT assay in SLN specimens. Meanwhile, blood-based multi-marker qRT assay is being validated in the phase III multicenter malignant melanoma active immunotherapy trial (MMAIT). Further studies incorporating circulating DNA assays are being conducted in the setting of phase II BC trials as well as in the MMAIT.

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