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# Fli-1 expression in malignant melanoma

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**Summary.** Friend leukemia integration site 1 (Fli-1) has been reported as the first nuclear marker of endothelial differentiation; it is expressed in leukocytes and recently demonstrated in melanomas. Formalin-fixed, paraffin-embedded tissue sections from 97 melanomas including 69 cases of primary and 28 metastatic melanomas were evaluated by immunohistochemistry. Five melanoma cell lines were evaluated by Western blot and immunocytochemistry. Fli-1 expression was observed in all cell lines. Fli-1 expression was higher in metastatic than in primary tumors (r=0.208, p=0.041, Spearman correlation), it positively correlated with Ki-67 expression (r=0.233, p=0.022, Spearman correlation), and the presence of an ulcer in the primary tumor (r=0.267, p=0.030, Spearman correlation). Therefore, the expression of Fli-1 in malignant melanoma appears to be associated with biologically more aggressive tumors.

**Key words**: Fli-1, Melanoma

## Introduction

Friend leukemia integration 1 transcription factor (Fli-1, Fli-1 proto-oncogene, ERGB transcription factor) is encoded by the *FLI1* gene which maps to 11q23-q24 (Baud et al., 1991; Hromas et al., 1993). Baud et al. (1991) showed that it lies on a fragment flanked on the centromeric side by the translocation breakpoint in acute lymphoblastic leukemia-associated t(4;11)(q21;q23) and on the telomeric side by the Ewing-associated t(11;22)(q24;q12) breakpoint. Human Fli-1 is a member of the ets family of transcription factors and is a sequence specific transcriptional activator recognizing the DNA sequence 5'-C(CA)GGAAGT-3'. The DNA-binding domain of human Fli-1 is 98 amino acid residues long (Liang et al., 1994a,b). The translocation of Ewing sarcoma t(11;22)(q24;q12) substitutes a putative RNA-binding domain of the Ewing sarcoma gene on chromosome 22 for the DNA-binding domain of the FLI1 gene on chromosome 11, which produces a chimeric transcription factor that requires the DNA-binding domain encoded by FLI1 for transformation (Delattre et al., 1992; May et al., 1993). Mouse Fli-1 was shown to be involved in 75% erythroleukemias induced by Friend murine leukemia virus suggesting the possibility that Fli-1 may play a critical role in cellular transformation (Prasad et al., 1992). Fli-1 was also established as an endothelial cell marker and can be used to demonstrate endothelial cell differentiation in malignant tumors, and as such it has been shown to be an excellent angiosarcoma marker (Folpe et al., 2001). Pusztaszeri et al. (2006) investigated the expression of various endothelial cell markers in normal human tissues and also found Fli-1 to be a relatively restricted endothelial cell marker. Otherwise, very little is known about the biology of Fli-1 protein in human diseases. Strong Fli-1 expression was first detected in a rare case of melanoma by Rossi and coauthors in 2004 (Rossi et al., 2004). These authors also observed that weaker Fli-1 expression is not unusual in melanoma, but concluded that the generally lower level of expression obtained in melanoma would not interfere in diagnostic practice, since endothelial lesions generally express high levels of this transcription factor. Therefore, Fli-1 continues to be considered as an excellent marker of angiosarcoma as originally described by Folpe and coauthors (Folpe et al., 2001), where its expression is related to the endothelial differentiation of the tumor cells. Nevertheless, Fli-1 is differentially expressed in melanoma. Also, this transcription factor is known to have an oncogenic potential when inappropriately expressed, and Fli-1 has been reported to participate in the regulation of the expression of genes such as Rb and Bcl-2, whose roles

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in malignancy have been well established (Tamir et al., 1999; Lesault et al., 2002). Direct transcriptional regulation of MDM2 by Fli-1 and its indirect downregulation of p53 were also shown (Truong et al., 2005). Our current study investigated whether the inappropriate expression of Fli-1 in malignant melanoma is associated with melanoma progression.

## Materials and methods

Formalin-fixed, paraffin-embedded tissue sections from 69 primary and 28 metastatic melanomas were randomly collected from the archives of the pathology department, The Norwegian Radium Hospital, Oslo, Norway, following the guidelines by the regional ethical committee. The median age of the patients was 62 years (range 19-93 years). Of the primary tumors, 54 were classified as superficial spreading and 15 as nodular. Ten control cases of benign nevi were also included.

### *Immunohistochemistry*

Formalin-fixed, paraffin-embedded tissue was cut at 5 µm, dried overnight at 60°C and deparaffinized in xylene. Subsequently, sections were rehydrated through graded alcohols into water. Heat-induced epitope retrieval was achieved by boiling sections in the EDTA buffer at pH 8.9 in an Electrolux microwave oven (Stockholm, Sweden) at 1000W for 20 minutes (4x5 min). After boiling, sections were allowed to cool at room temperature for 20 minutes, rinsed thoroughly with water and placed in Tris-buffered saline (TBS) for 5 minutes. Endogenous peroxidase was blocked with Peroxidase Block solution provided in the EnVision+® kit (Dako, Glostrup, Denmark) for 5 minutes and slides rinsed/washed with TBS. Slides were then incubated with 1G11 antibody (1:20, NovoCastra Laboratories, New Castle upon Tine, UK) for 30 minutes at room temperature. Visualization was performed using EnVision+® (Dako, Glostrup, Denmark) method according to the manufacturer's instructions. Appropriate positive and negative controls were used. In addition, strong nuclear staining in benign small lymphocytes was used as an internal positive control. Only nuclear staining was recorded. Expression of Fli-1 was quantified using the H-score (histo-score) system, according to the method described previously (McCarty et al., 1985), which considers both the intensity and percentage of cells staining at each intensity. Cells were counted by using 400X magnification and a cell counter. The score was calculated as follows: H-score = (% 3+ cells X 3) + (% 2+ cells X 2) + (% 1+ cells X 1). Also, the overall percentage of positive cells was recorded, as well as the pathologist's semiquantitative estimate of the overall staining intensity (0-3+). The cases were also designated as "positive" if more than 10% of the tumor cells showed expression of the antigen. The tumors were also immonostained by anti-Ki-67 antibody as described previously (Bilalovic et al., 2004).

### *Cell cultures*

All human melanoma cell lines used in this study were routinely cultured in RPMI 1640 medium (BioWhittaker Europe, Verviers, Belgium) supplemented with 5% fetal bovine serum (FBS) (Biochrom KG, Berlin, Germany). Cell lines used in the study were: WM35, WM1341B, WM45.1, WM239, FEMX-I, and Jurkat. The cell lines of the WM series were kindly provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA, USA) and have been described in detail elsewhere (Cornil et al., 1991). The FEMX-1 cells were established from a lymph metastasis (Fodstad et al., 1988). The Jurkat cell line was obtained from American Tissue Cell Collection (ATCC).

### *Immunoblotting*

Cells were lysed in ice-cold NP-40 lysis buffer (1% NP-40, 10% glycerol, 20 mM Tris-HCl pH 7.5, 137 mM NaCl, 100 mM sodium vanadate, 1 mM phenylmethyl sulphonyl fluoride (PMSF) and 0.02 mg/ml each of aprotinin, leupeptin and pepstatin and 10 l/ml of phosphatase inhibitor coctail I). All protease inhibitors were from Sigma Aldrich (Sigma-Aldrich, St. Louis, Mo). Lysates were sonicated and clarified by centrifugation. Protein quantitation was done by Bradford analysis and 25 g protein/lane was resolved by SDS- polyacrylamide-gel-electrophoresis. Transfer and hybridization were performed as described previously (Dulic et al., 1992).

### *Statistical methods*

The chi-square, linear-to-linear association and Mann-Whitney U tests were used whenever appropriate for comparison of subgroups. The association between the intensity of Fli-1 expression recorded by the H-score with proliferation rate (percent Ki-67-positive cells), primary or metastatic tumors, depth of invasion (Clark levels); tumor thickness according to Breslow, and diameter of tumors was studied with the Spearman Rank Correlation. Linear regression was used to compare explanatory variables. Statistical significance was established at the $p<0.05$ level. Analyses were performed in SPSS 11.5.

## Results

When 10% positive cells was used as a cut off point, nuclear Fli-1 expression was found in 48% of primary and 57% of metastatic melanomas. Overall, 50% were negative, 32% weakly positive, 14% were moderately positive (2+), and 4% were strongly positive (3+) (Table 1). Ten control nevi did not express Fli-1. When the intensity of Fli-1 expression was recorded by employing the H-score method, it was significantly higher in metastatic melanoma ($r=0.208$, $p=0.041$, Spearman correlation) with an overall mean score of 88 (range 0 -

242) (Figs. 1-3). No difference between the superficial spreading and nodular melanoma were found. Also, there was no difference between the lesions occurring on arms and legs vs. more axial lesions.

Overall, the Fli-1 H-score also showed positive correlation with the percentage of tumor cells positive for Ki-67 protein, indicating its association with increased proliferation in melanoma cells (r=0.233, p=0.022, Spearman correlation) (Table 2, Fig. 2). Although the proliferation rate was higher in metastatic tumors (p=0.018, Mann-Whitney U test), when primary tumors were analyzed separately, a statistical trend for a positive correlation between Fli-1 expression and Ki-67 expression was still present (r=0.214, p=0.078, Spearman correlation).

In primary tumors, the presence of ulceration showed a positive association with higher age (p=0.011), depth of tumor invasion (p=0.004), and Clark levels (p=0.012) (Table 2). Fli-1 expression also had a positive correlation with the presence of an ulcer (r=0.267, p=0.030, Spearman correlation) (Table 2). Since the presence of ulceration was in itself strongly positively associated with a higher percentage of Ki-67-positive cells (p<0.0001, Mann-Whitney U test) the analyses were therefore repeated after excluding cases with ulceration. Again, Fli-1 expression was found to be significantly higher in metastatic tumors than in primary tumors without ulceration (r=0.365, p=0.001, Spearman Correlation); the difference between the primary and metastatic tumors being even more obvious than when cases of primary tumors with ulceration were also included in the analysis. Also, when cases with ulceration were excluded, the positive statistical trend with Ki-67 expression remained nevertheless (r=216,

**Table 1.** Fli-1 Expression in Primary and Metastatic Malignant Melanoma.

| | Fli-1 (%) | | | | Total |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | |
| Primary Melanoma | 36 (52) | 23 (33) | 8 (12) | 2 (3) | 69 (100) |
| Metastatic Melanoma | 12 (43) | 8 (29) | 6 (21) | 2 (7) | 28 (100) |
| Total | 48 (50) | 31 (32) | 14 (14) | 4 (4) | 97 (100) |

**Table 2.** Fli-1 expression in relation to melanoma proliferation, ulceration and Clark levels.

| | Fli-1 Expression (%) (H-score Median =62) | |
|---|---|---|
| | H-Score <62 | H-Score >62 |
| Ki-67 Proliferation Rate (N=97) | | |
| Negative | 4 (4) | 2 (2) |
| < 5% cells | 13 (13) | 11 (12) |
| >5%-20% | 15 (16) | 19 (19) |
| >20%-50% | 10 (10) | 16 (17) |
| >50% | 0 | 7 (7) |
| Ulceration (Primary Only, N=68) | | |
| No | 29 (42) | 23 (34) |
| Yes | 4 (6) | 12 (18) |
| Clack levels (Primary Only, N=68) | | |
| 2 | 8 (12) | 4 (6) |
| 3 | 10 (15) | 13 (19) |
| 4 | 9 (13) | 8 (12) |
| 5 | 6 (9) | 10 (14) |

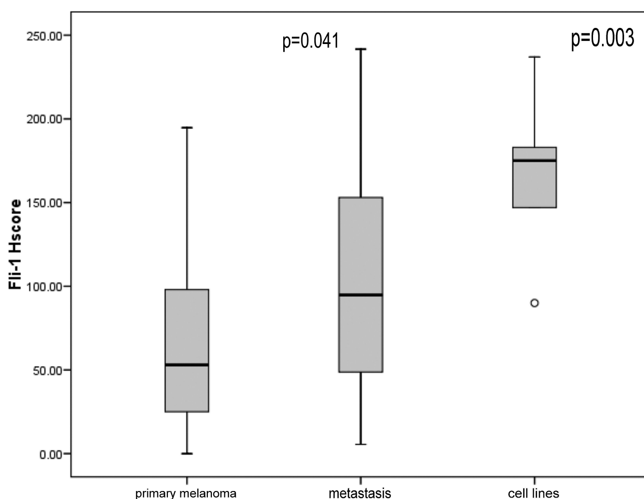


**Fig. 1.** Fli-1 expression was higher in metastatic than in primary melanomas (r=0.208, p=0.041, Spearman Correlation), but overall highest expression was found in melanoma cell lines (r=0.293, p=0.0003, Spearman Correlation).

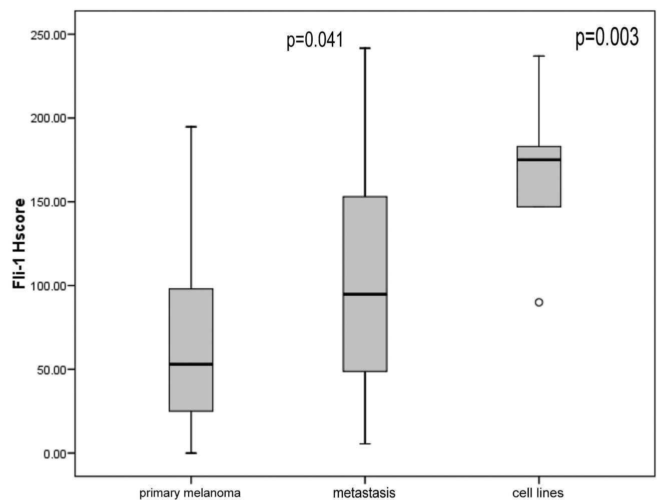


**Fig. 2.** There was a positive association between Fli-1 expression and proliferation rate as measured by Ki-67 expression in tumor cells (r=0.233, p=0.022, Spearman correlation).

p=0.053, Spearman Correlation). One case of the primary melanoma was exluded from the analysis regarding the presence of ulceration since it was not possible to determine with certainity from clinical data and histologic slides whether the ulceration was present or not. Similarly, in one case of primary tumor, it was not possible to determine with certainty the exact Clark level and therefore 68 primary tumors were analyzed with respect to this variable.

Linear regression analysis suggested that the metastatic tumors were independently associated with a higher proliferation rate and higher Fli-1 expression, rather than proliferation rate being independently associated with Fli-1 expression because Fli-1 was not a statistically significant explanatory variable for the

**Table 3.** Fli-1 expression in melanoma cell lines.

| Cell line | Immunocytochemistry | | Western Blot |
|---|---|---|---|
| | H-Score* | % Positive Cells | |
| WM35 | 147 | 97 | + |
| WM239 | 90 | 67 | ++ |
| WM1341B | 175 | 94 | + |
| WM45.1 | 237 | 100 | ++ |
| FEMX-I | 183 | 99 | -/+ |

* H-Score range is 0 to 300.

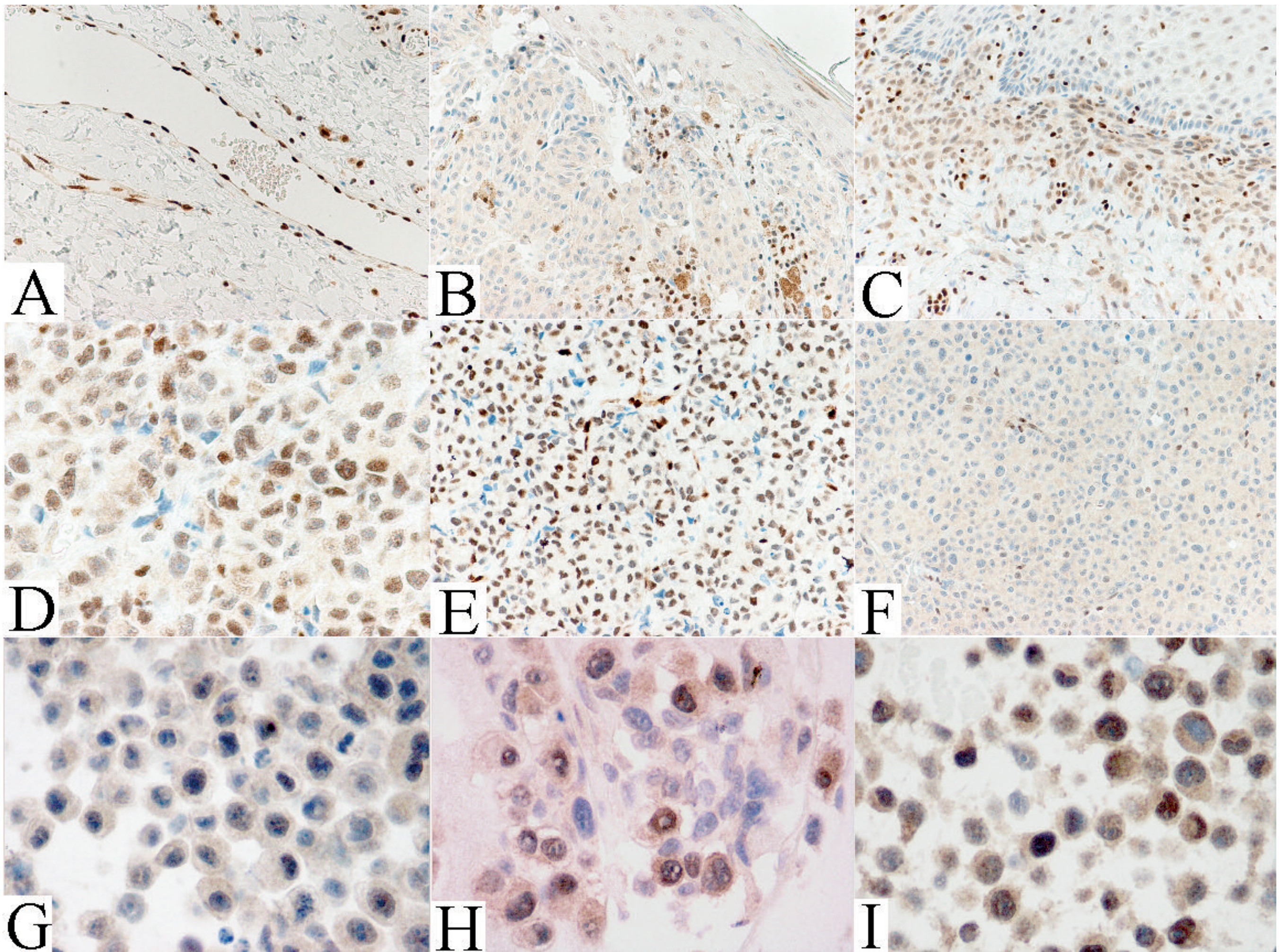


**Fig. 3.** Fli-1 is readily detected in endothelial cells in various tissues **(A)**. Many primary melanomas do not express Fli-1 **(B)**, but Fli-1 can be found generally weakly expressed in primary melanoma, frequently in cases with ulceration **(C)**. Although Fli-1 expression was higher in metastatic melanoma, most positive cases showed variable expression from cell to cell **(D)** and only rare cases had strong, uniform expression **(E)**. Almost half of the metastatic melanomas were completely negative **(F)**. While Fli-1 expression also varied in different melanoma cell lines, its expression was overall higher in cell lines than in patients' samples **(G-I)**. A-C, E, F, x 200; D, G-I, x 400

percentage of Ki-67-positive cells.

No association was found with the depth of invasion as measured by either Clark's levels or by Breslow thickness (Table 2).

Fli-1 was also expressed in all five melanoma cell lines tested (Table 3). Overall, the expression of Fli-1 was significantly higher in cell lines than in primary or even metastatic melanomas (r=0.293, p=0.003, Spearman Correlation).

## Discussion

Fli-1 is a nuclear transcription factor involved in cellular proliferation and tumorigenesis. Its role has been investigated in Ewing sarcoma and primitive neuroectodermal tumors, where it is implicated due to the presence of a specific translocation t(11;22) in 90% of the cases, resulting in the fusion of the EWS gene on chromosome 22 to the *Fli-1* gene on chromosome 11 (Hahm et al., 1999; Nakatani et al., 2003). The potential role of Fli-1 in melanoma progression probably does not bear any similarity to its role in Ewing sarcoma. Hahm et al. (1999) hypothesized that in Ewing sarcoma, TGFBR2 may be a target of the EWS/FLI1 fusion protein and showed that embryonic stem cell lines with the EWSR1-FLI1 fusion have reduced TGF-beta sensitivity, and that fusion-positive ES cells and primary tumors both express low or undetectable levels of TGFBR2 mRNA and protein product. In addition to its effects on TGTBR2, reporter gene assays indicated that the binding of EWS-FLI1 to at least 2 ETS binding sites negatively regulated p21(WAF1) promoter activity. EWS-FLI1 also suppressed p21(WAF1) induction by interacting with p300 and inhibiting its histone acetyltransferase activity (Nakatani et al., 2003).

The oncogenic potential of Fli-1 has been demonstrated in erythroblastic leukemia (Pereira et al., 1999; Lesault et al., 2002). Fli-1 was shown to induce the proliferation of differentiation-arrested erythroblasts with enhanced survival of Fli-1 expressing erythroblasts correlating with upregulated *BCL2* expression (Pereira et al., 1999). A separate study found that direct regulation of BCL2 by Fli-1 is involved in the survival of Fli-1-transformed erythroblasts (Lesault et al., 2002). In our study, Fli-1 expression in malignant melanoma was found to be associated with a higher proliferation rate of the neoplastic cells. Fli-1 expression was also previously found in Merkel cell carcinoma (Llombart et al., 2005). Despite only 20 cases studied, it showed a statistical trend for a positive association with Ki-67.

Strong expression of Fli-1 was demonstrated by Rossi et al. (2004) in 1/10 melanomas and weak expression in an additional 5/10 cases. Our study is the first to evaluate Fli-1 expression in a larger series of malignant melanoma or in melanoma cell lines. The somewhat higher percentage of strongly positive cases may reflect inclusion of metastatic melanomas, which in general tended to show higher expression of Fli-1 than primary tumors. Interestingly, while Fli-1 protein was expressed in higher levels in metastatic melanomas, the highest levels in our study were detected in melanoma cell lines. While Fli-1 is normally expressed in endothelial cells and its expression can be used as evidence of endothelial differentiation, potential proto-oncogenic properties of inappropriately expressed Fli-1 protein should be further evaluated. Fli-1 has so far been demonstrated to be aberrantly expressed in Merkel cell carcinoma (Llombart et al. 2005) where, just like in our study, it appeared to have a positive association with proliferation index as determined by Ki-67 expression.

Our study confirms differential and inappropriate expression of Fli-1 transcription factor in malignant melanoma, with higher levels found in ulcerated primary tumors, metastatic tumors, and overall with melanomas with higher proliferation rate. Further studies are required to elucidate the underlying biological mechanisms.

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