Stem Cell Marker Nestin and c-Jun NH_2-Terminal Kinases in Tumor and Peritumor Areas of Glioblastoma Multiforme: Possible Prognostic Implications

Annunziato Mangiola,[1] Gina Lama,[2] Cecilia Giannitelli,[2] Pasquale De Bonis,[1] Carmelo Anile,[1] Libero Lauriola,[3] Giuseppe La Torre,[4] Giovanni Sabatino,[1] Giulio Maira,[1] Meena Jhanwar-Uniyal,[5] and Gigliola Sica[2]

Abstract

Purpose: It has been hypothesized that brain tumors are derived from stem cell or transiently dividing precursor transformation. Furthermore, c-Jun NH_2-terminal kinases (JNKs) have been involved in gliomagenesis. This study analyzes stem cell marker nestin and JNK expression in glioblastoma multiforme (GBM) and peritumor tissue and assesses their possible prognostic implications.

Experimental Design: Nestin and both total JNK (tJNK) and phosphorylated JNK (pJNK) expression was investigated by immunohistochemistry in 20 GBMs. Samples were derived from tumors (first area), from tissues at a distance ‹1 cm (second area), and between 1 and 3.5 cm (third area) from the macroscopic tumor border. The relationships between patients' age, Karnofsky performance status, gender, protein expression, and survival were analyzed.

Results: Nestin cytoplasmic immunoreactivity was observed in the majority of cells in tumor but infrequently in peritumor areas. tJNK, observed in the nucleus and cytoplasm, was widely expressed in the three areas; pJNK, mostly located in the nuclei, was found in a variable percentage of cells in the tumor and peritumor tissue. Nestin and JNK expression in peritumor areas was independent of the presence of neoplastic cells. Univariate analysis indicated that survival was longer (19 versus 12 months; $P = 0.01$) for patients whose pJNK/nestin and (pJNK/tJNK)/nestin ratios in the second area were $\geq$2.619 and $\geq$0.026, respectively. The same variables showed an independent prognostic value in multivariate analysis.

Conclusions: Nestin and JNK expression indicates that peritumor tissue, independently of the presence of neoplastic cells, may present signs of transformation. Moreover, pJNK/nestin and (pJNK/tJNK)/nestin ratios in that tissue seem to have some prognostic implications in GBM patients.

Gliomas represent the most common primary brain tumors. Glioblastoma multiforme (GBM), its most malignant form, is aggressive, highly invasive, and neurologically devastating (1).

Specific treatment includes surgery, radiotherapy, and chemotherapy, but GBM remains incurable despite the technological advances and novel therapeutics. The median survival time is ~1 year after diagnosis (2) and the overall 5-year survival rate is ~2% (3).

Alterations in adult neurogenesis might contribute in the development of GBM, which may derive from the transformation of neural stem cells or transiently dividing progenitors (4). It has been reported that mature and immature elements may be differentiated based on the expression of nestin, a class VI intermediate filament (5). This protein is expressed in undifferentiated cells and radial glia during the developing mammalian brain, but the differentiation of neural stem cells/progenitor cells into postmitotic astrocytic and neural cells is accompanied by the down-regulation of nestin and the expression of other types of intermediate filaments (6). Reexpression of down-regulated nestin has been shown in reactive astrocytes following brain injuries and cerebral ischemia (7, 8).

Nestin has been detected in a variety of primary human brain tumors, including glioblastomas (9, 10). This supports the stem cell hypothesis, which could be linked to the tumor radiation resistance and repopulation (11).

The genetic aberrations necessary for stem cell and progenitor cell transformation are being uncovered (12), whereas in the full-blown tumors a wide series of alterations of tumor

Authors' Affiliations: [1]Institute of Neurosurgery, [2]Institute of Histology and Embryology, [3]Institute of Pathology, and [4]Unit of Epidemiology and Biostatistics-Institute of Hygiene, Faculty of Medicine, Catholic University of the Sacred Heart, Rome, Italy and [5]Departments of Neurosurgery and Experimental Pathology, New York Medical College, Valhalla, New York

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Requests for reprints: Meena Jhanwar-Uniyal, Department of Neurosurgery, New York Medical College, Valhalla, NY 10595. Phone: 914-594-3186; E-mail: Meena_Jhanwar@nymc.edu or Gigliola Sica, Institute of Histology and Embryology, Faculty of Medicine, Catholic University of the Sacred Heart, Largo Francesco Vito 1, 00168 Rome, Italy. Phone: 39-06-3053261; Fax: 39-06-3053261; E-mail: ibiis@rm.unicatt.it.

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suppressor genes and oncogenes has been assessed (13). Moreover, recent investigations suggest that GBMs can be subclassified based on their gene expression profile (14). In this context, genes encoding for growth factors and growth factor receptors seem to play an important role and can be used for patient stratification to determine prognosis and treatment (15).

The signal transduction pathways activated by growth factors include mitogen-activated protein kinases, a family comprising the extracellular signal-regulated kinases (ERKs), stress-activated protein kinases/c-Jun NH_2-terminal kinases (JNKs), and p38 mitogen-activated protein kinases (16).

ERKs are mainly involved in the regulation of cell proliferation, differentiation, and motility (17, 18). Furthermore, several studies have shown the significance of ERK expression and their prognostic relevance in GBM (19–21). Recently, our group reported the presence of activated ERKs not only in the enhanced lesion but also in the peritumor tissue (22).

Activated JNK has been found in rat glial cells after traumatic injuries (23) and in reactive astrocytes in a model of temporal lobe epilepsy (24). A possible JNK meaning has been suggested in the acquisition of the transformed phenotype. In fact, JNK activation is required for cellular transformation by certain oncogenes (25). Nevertheless, it is commonly thought to regulate apoptosis (26).

In the present study, we evaluated by immunohistochemistry the expression of nestin as well as total JNK (tJNK) and activated JNK (pJNK) in samples obtained from primary GBM and from the peritumor tissue up to 3.5 cm from the macroscopic margin of the neoplasia.

The primary aim of our investigation was to verify nestin and JNK location. In addition, protein expression in relation to the presence of neoplastic cells in peritumor areas was evaluated. Furthermore, univariate and multivariate analyses were used to establish a possible correlation between both nestin and JNK expression and prognosis.

Materials and Methods

Patients and tissue samples. This study involved 20 adult patients with supratentorial GBM who underwent "en bloc" surgery at the Catholic University in Rome between October 2002 and November 2005.

Based on the patients' clinical history, the GBMs were considered as primary. In fact, the time interval between first signs of disease and hospital admission was very short in all cases and the patients had a rapid clinical progression of disease. Moreover, the age distribution curve of our population fits that of primary GBMs. Nonetheless, we cannot exclude the presence of secondary GBMs with rapid onset progression of symptoms among our cases.

The clinicopathologic characteristics of the patients are reported in Table 1. Ten patients were males and 10 were females, with a mean age of 60.45 years (range, 43-72). All patients had a Karnofsky performance status (KPS) of $\geq$70.

Thirty-five days after surgery (range, 30-40 days), patients received external source-limited field radiotherapy (average dose of 60 Gy administered in fractions). Simultaneously, chemotherapy with temozolomide (marketed as Temodal in Europe and in Canada and Temodar in the United States) was administered at a dose of 75 mg/m^2/d for 7 days per 6 weeks. After a 1-month break, patients received up to six cycles of temozolomide at a dose of 150 to 200 mg/m^2/d according to the standard of 5 days per week every 28 days.

Survival time was computed from the date of surgery to the date of death (or to December 31, 2006 for one patient still alive).

The adopted surgical technique allowed us to obtain samples from the enhanced lesion (first area) and white matter at a distance <1 cm (second area) and between 1 and 3.5 cm (third area) from the macroscopic tumor border. The larger resections were done in tumors growing far from eloquent areas. Nevertheless, neoplasm anatomic location did not always allow us to obtain samples from the three areas in all cases considered: 20 tissue specimens were derived from the first, 18 from the second, and 15 from the third area.

Tissue samples from each zone were fixed in 10% neutral buffered formalin immediately after excision. Specimens were histologically assessed using H&E sections by a single pathologist. Neoplastic cells were identified by their nuclear atypia and heteropyknotic staining.

Table 1. Clinicopathologic characteristics of the 20 adult patients with GBM (IV WHO)

Patients	Sex	Age at diagnosis (y)	Tumor localization	KPS score	Treatment	Survival time (mo)	Clinical outcome
1	M	67	Parietotemporal	80	CH+RT	8	DOD
2	M	63	Frontal	100	CH+RT	11	DOD
3	F	47	Frontal	100	CH+RT	14	DOOC
4	F	65	Parietooccipital	80	CH+RT	18	DOD
5	F	58	Frontal	100	CH+RT	15	DOD
6	F	71	Frontal	80	CH+RT	13	DOD
7	F	61	Frontal	100	CH+RT	12	DOD
8	M	58	Parietotemporal	80	CH+RT	19	DOD
9	M	72	Parietal	90	CH+RT	12	DOD
10	M	43	Parietal	80	CH+RT	19	DOD
11	F	68	Frontal	90	CH+RT	28	DOD
12	F	49	Frontal	100	CH+RT	35	Alive
13	F	47	Frontal	100	CH+RT	25	DOD
14	F	65	Frontal	90	—	2	DOOC
15	M	69	Frontal	70	—	0.1	DOOC
16	M	71	Parietal	80	CH+RT	22	DOD
17	M	61	Frontotemporal	100	CH+RT	13	DOD
18	F	62	Frontoparietal	80	CH+RT	14	DOD
19	M	68	Parietotemporal	100	CH+RT	12	DOD
20	M	44	Frontal	100	CH+RT	19	DOD

Abbreviations: M, male; F, female; DOD, dead of disease; DOOC, dead of other causes; CH, chemotherapy; RT, radiotherapy.

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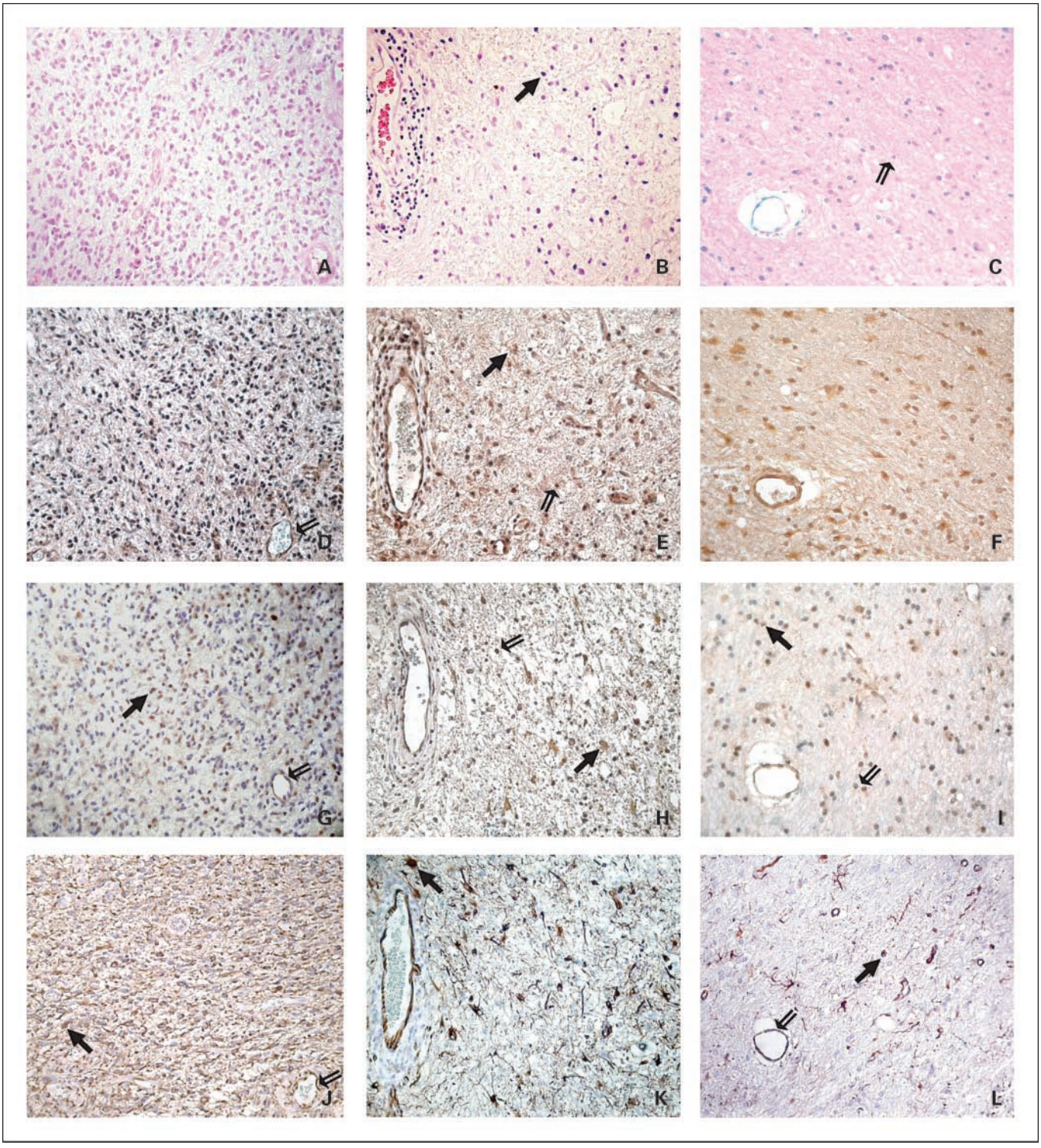

Fig. 1. Localization of nestin, tJNK, and pJNK in GBM and peritumor tissue. Serial sections from first (*A, D, G*, and *J*), second (*B, E, H*, and *K*), and third (*C, F, I*, and *L*) areas. *A,* GBM with high cellularity, cellular anaplasia, and vascular proliferation (H&E staining). *D,* nestin immunoreactivity was observed in the cytoplasm of the neoplastic cells. Small arrow, immunolabeling was detected in the endothelium of the vessel wall. *G,* a high number of tumor cells exhibited tJNK immunoreactivity, located both in the nucleus and in the cytoplasm. Small arrow, endothelium was also immunostained. *J,* tumor cells exhibited pJNK immunoreactivity mostly in the nuclei. Small arrow, endothelial cells were immunolabeled. *B,* black arrows, in a second area, some cells with moderately irregular and atypical nuclei that are suspected for neoplasia were present (H&E staining). *E,* atypical cells (*black arrow*), reactive astrocytes (*head arrow*), and normal cells (*open arrow*) exhibited nestin immunolabeling in their cytoplasm. Small arrow, endothelial cells of hyperplastic vessels were immunolabeled also. *H,* atypical cells (*black arrow*), reactive astrocytes (*head arrow*), and apparently normal cells (*open arrow*) showed tJNK immunoreactivity. *K,* pJNK immunoreactivity was observed in cells with atypical features (*black arrow*), in reactive astrocytes (*head arrow*), and in apparently normal cells (*open arrow*). *C,* in a third area, apparently normal cells with small round nuclei and scanty cytoplasm were present (H&E staining). *F,* both reactive astrocytes (*head arrow*) and apparently normal cells (*open arrow*) showed nestin immunoreactivity that was also observed in endothelial cells of the microvessels (*small arrow*). *I,* both reactive astrocytes (*head arrow*) and apparently normal cells (*open arrow*) exhibited tJNK immunoreactivity. *L,* pJNK immunoreactivity was observed in reactive astrocytes (*head arrow*) as well as in apparently normal oligodendroglial-like cells (*open arrow*). Original magnification, ×200 (*A-N*). Hematoxylin counterstain.

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Reactive astrocytes were recognized by dendritic morphology of their abundant eosinophilic cytoplasm and large eccentric nuclei according to Hoelzinger et al. (27). The classification and grading of the tumors were done according to the criteria of the WHO (28). All patients gave consent to use their tumor and peritumor material as well as clinical data for research purpose. It is worth mentioning that 15 of 20 patients were analyzed for both nestin and JNKs. About the remaining 5 patients, 4 of them were analyzed only for nestin and 1 only for JNKs. In total, nestin was determined in 19 of 20 patients, whereas tJNK and pJNK expression was determined in 16 of 20 patients.

Immunohistochemistry. Immunohistochemistry was carried out on 5-μm-thick sections on polylysine-coated slides. After routine deparaffinization and rehydration, antigen retrieval was done. Slide-mounted sections were heated in a microwave oven at 600 W thrice for 5 min or boiled in 10 mmol/L sodium citrate buffer (pH 6.0) for 15 min for nestin and JNKs, respectively. Tissue sections were allowed to cool at room temperature. Quenching of endogenous peroxidase activity was done either in methanol containing 3% H_2O_2 for nestin or in TBS/Triton X-100 (pH 7.4) containing 2% H_2O_2 for JNKs.

Anti-nestin mouse monoclonal antibody (clone 10C2; 1:200 diluted; Chemicon International); anti-JNK2 (FL) rabbit polyclonal antibody (1:50 diluted; Santa Cruz Biotechnology, Inc.) and anti-phospo-JNK monoclonal antibody (clone G-7; 1:50 diluted; Santa Cruz, Biotechnology) which recognize the JNK1, JNK2, and JNK3 isoforms, were used. After washings, the slides were incubated with the secondary biotinylated goat anti-mouse and anti-rabbit IgG antibodies (both 1:300 diluted; Vector Laboratories, Inc.) and subsequently with the avidin-biotin peroxidase complex (Elite ABC-peroxidase kit, Vector Laboratories).

The chromogenic reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride solution (Peroxidase DAB substrate kit, Vector Laboratories). The nuclei were lightly counterstained with Mayer's hematoxylin. In negative controls, where primary antibodies were omitted, specific immunoreactivity was not detected.

Human GBM specimens and normal human skin were used as positive controls for nestin and both anti-JNK2 and anti-phosphorylated JNK antibodies, respectively.

Two sections of each area were examined by two independent observers and at least 1,000 to 2,500 cells from 4 to 10 randomly selected fields in each section were counted at ×400 magnification using a light microscope (Axioskop 2 Plus, Zeiss). The few cases with discrepant scoring were reevaluated jointly and agreement was reached.

The percentage of positive cells was calculated and reported as average ± SD.

All immunolabeled cells, excluding the endothelial ones, were counted.

Statistical analysis. The statistical analyses were carried out using Statistical Package for the Social Sciences software package for Windows (version 11.0.1; SPSS, Inc.). The Kruskal-Wallis test was used for comparing the levels of nestin or JNK expression among the three areas. To find differences in the levels of each protein expression between two areas (first versus second area, first versus third area, and second versus third area), the Mann-Whitney (unpaired data) and the Wilcoxon signed rank (paired data) tests were used.

Patients' survival rates were determined by the Kaplan-Meier method. The correlation between age groups (<65 and ≥65 years), KPS (KPS = 80 and KPS = 90-100), gender, biological variables, and survival was analyzed using the log-rank test. In particular, patients were dichotomized into two groups, considering the median value of each biological variable as the cutoff point. For those factors showing an association with survival in univariate analysis, Cox proportional hazards regressions were fitted to evaluate the effect of each variable on survival, adjusting for potential confounders such as known prognostic factors (age and pre-operative KPS). Statistical significance was set at $P < 0.05$ in all tests.

Results

Expression of nestin, tJNK, and pJNK in tumor and peritumor areas. In the tumor area, typical features of marked hypercellularity, nuclear atypia, frequent mitoses, and vascular proliferation were observed (Fig. 1A). In peritumor areas, characterized by a decrease in cell density, apart from apparently normal glial cells and reactive hypertrophic astrocytes, neoplastic-appearing cells were found in different percentages (up to 50%) or they were completely absent (Fig. 1B and C).

Nestin immunoreactivity, mostly localized in the cytoplasm, was detected in the majority of cells in the tumor (Table 2; Figs. 1D and 2A) but it was scarcely expressed in the peritumor tissue (Table 2). In the second and third areas, it was present in few cells, including neoplastic elements, reactive glial cells, and apparently normal cells (Fig. 1E and F). In particular, in some glial cells, cytoplasmic extensions were positive (Fig. 2B). Moreover, nestin was expressed not only by endothelial cells of hyperplastic vessels (Fig. 1D and E) but also by microvessel endothelial cells in peritumor areas (Fig. 2B).

Nestin expression was lower in the second and third areas when compared with the first (Table 2). The Kruskal-Wallis test showed a difference in nestin expression among the three areas ($P < 0.0001$). The Mann-Whitney test indicated a difference between the first and the second areas ($P < 0.0001$) and between the first and the third areas ($P < 0.0001$). Similarly, the Wilcoxon signed rank test showed a difference between the first

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Table 2. Nestin, tJNK, and pJNK expression in the enhanced lesion and in the peritumor areas of GBM patients

		First area	Second area	Third area
Nestin-positive cells (%)	Mean ± SD	85.58 ± 10.28	15.47 ± 7.74	13.48 ± 9.44
	Median	87.14	13.35	11.50
	Range	71.37-98.68	7.98-39.14	2.97-40.37
No. samples		19	17	14
tJNK-positive cells (%)	Mean ± SD	94.44 ± 6.75	97.98 ± 3.36	96.67 ± 8.56
	Median	97.77	99.29	99.31
	Range	80.32-99.45	88.67-100	68.33-99.95
No. samples		16	14	13
pJNK-positive cells (%)	Mean ± SD	72.25 ± 25.75	54.53 ± 30.89	54.21 ± 33.59
	Median	73.13	48.31	35.19
	Range	18.73-99.49	14.72-99.15	13.26-98.97

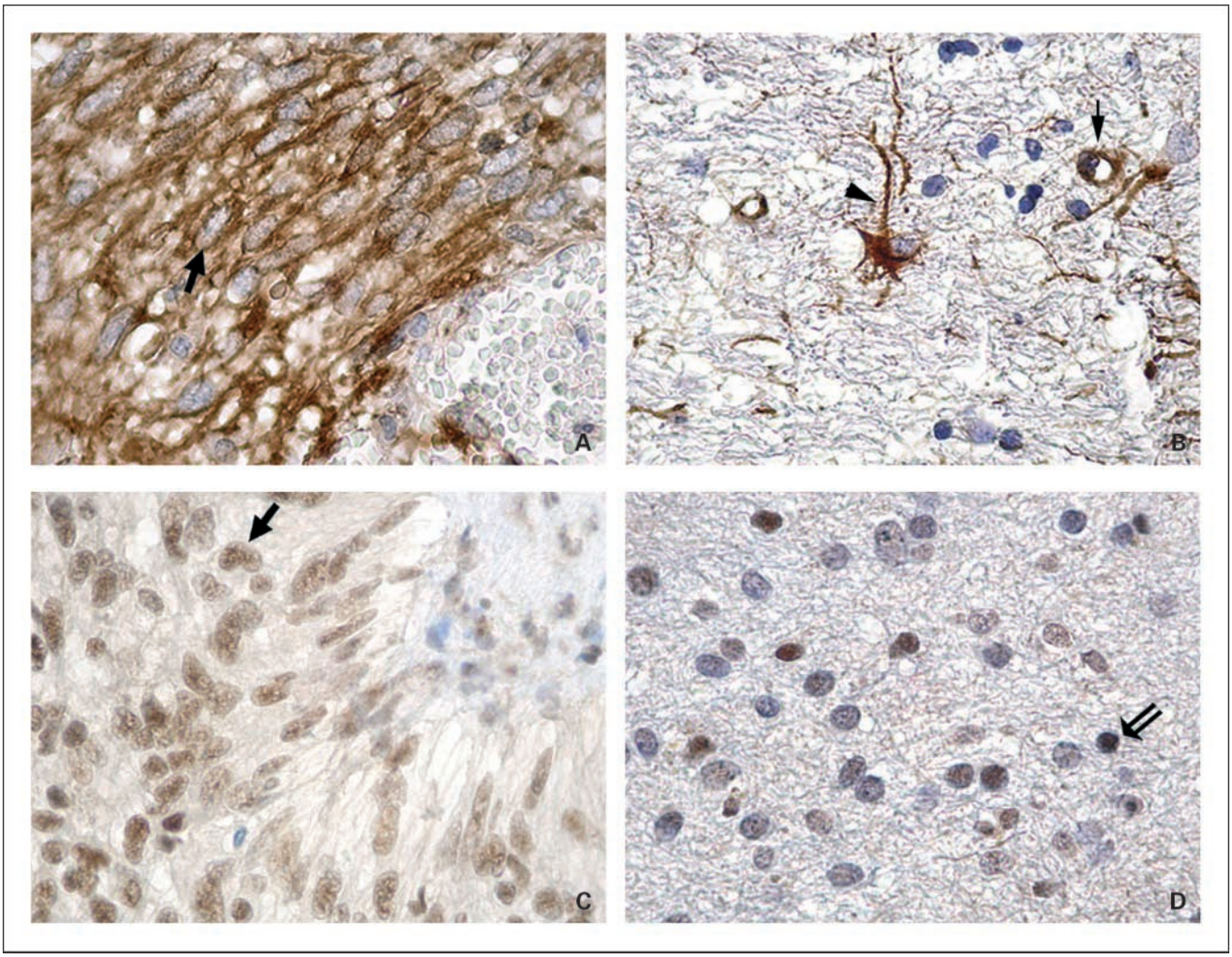

Fig. 2. Features of nestin and pJNK expression in GBM and peritumor areas. *A,* in a first area, the majority of neoplastic cells exhibited perinuclear and cytoplasmic (*black arrow*) nestin expression. *B,* small arrow, in a peritumor area (third area), nestin immunoreactivity was evident in the endothelium of microvessels; head arrow, a reactive astrocyte showed positive immunoreactivity including in the cytoplasmic extensions. *C,* black arrow, in a first area, pJNK immunoreactivity was present in the nucleus of neoplastic cells. *D,* open arrow, in a peritumor area (third area), pJNK immunoreactivity was evident in the nucleus of an apparently normal cell. Original magnification, ×630 (*A-D*). Hematoxylin counterstain.

and the second ($P < 0.0001$) areas and between the first and the third ones ($P = 0.001$). No differences were found between the second and the third areas. The Mann-Whitney test showed no difference in nestin expression in the two peritumor areas in relation to the presence or absence of tumor cells ($P = 0.743$ and 0.628, respectively).

tJNK, as well as pJNK, staining was seen not only in specimens taken from the tumor but also in the peritumor tissue (Table 2). tJNK was detected both in the nucleus and in the cytoplasm of most neoplastic cells, reactive glial cells, and apparently normal cells (Fig. 1G-I). pJNK, mainly located in the nucleus, was present in a lower number of neoplastic cells in the tumor (Figs. 1J and 2C), reactive astrocytes, and apparently normal cells (Figs. 1K and L and 2D). Both total and phosphorylated immunoreactivity was found in endothelial cells in the tumor and peritumor areas (Fig. 1G-L).

tJNK expression was higher in the second and third areas in comparison with the first one (Table 2). The Kruskal-Wallis test showed a difference in tJNK expression among the three areas ($P = 0.035$). Similarly, the Mann-Whitney test indicated a difference between the first and the second areas ($P = 0.025$) and between the first and the third areas ($P = 0.032$), whereas Wilcoxon signed rank test indicated a difference in tJNK expression only between the first and the second areas ($P = 0.002$). Both Mann-Whitney and Wilcoxon signed rank tests showed no differences between the second and the third areas.

Our results globally showed that differences in tJNK expression are present not only among the areas considered together but also in the different areas of the same subject. Moreover, the Mann-Whitney test did not reveal any difference in the two peritumor areas in relation to the presence or absence of tumor cells, suggesting that neoplastic cells did not influence their expression ($P = 0.491$ and 0.937, respectively).

pJNK expression was variable in the tumor and in the two peritumor areas (Table 2). The Kruskal-Wallis test showed no differences in pJNK expression among the three areas ($P = 0.194$). The Mann-Whitney and the Wilcoxon signed rank tests showed that no significant difference existed in the expression

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of pJNKs between the first and the second areas ($P = 0.131$ and 0.109, respectively) or between the first and the third areas ($P = 0.132$ and 0.196, respectively). In addition, no difference existed between the second and the third areas ($P = 0.943$ and 0.213, respectively). The Mann-Whitney test showed no difference in pJNK expression in the two peritumor areas in relation to the presence or absence of tumor cells ($P = 0.059$ and 0.240, respectively).

Survival analysis. The Kaplan-Meier analysis was done on 18 patients because patient nos. 14 and 15 (Table 1), who did not receive radiochemotherapy, were excluded. The analysis indicated that age groups (<65 and ≥65 years), KPS, and gender were not associated with survival time.

No association between nestin and tJNK expression in the three areas with survival was found. A trend toward significance was noticed for pJNKs in the second area: the median survival time was longer for patients with values ≥48.31% compared with patients expressing lower pJNK values (19 versus 12 months; $P = 0.056$).

To use the information of more than one variable, the prognostic potential value of ratios between variables was analyzed; pJNK/nestin and pJNK/tJNK ratios were calculated for each patient and the median value was used as the cutoff. Moreover, the ratio between pJNKs and tJNKs was calculated and, in turn, divided by the correspondent nestin value. The median value of (pJNK/tJNK)/nestin ratios was used as cutoff.

All results of these analyses are summarized in Table 3.

A trend toward significance for pJNK/tJNK ratio in the second area was found: the median survival time was longer for patients with values ≥0.484 (19 versus 12 months; $P = 0.056$) compared with patients expressing lower values. Moreover, pJNK/nestin and (pJNK/tJNK)/nestin ratios in the second area

Table 3. Results of Kaplan-Meier analysis giving probability values obtained with the log-rank test in the enhanced lesion and in the peritumor areas of GBM patients

		No. samples	Cutoff	No. cases dichotomized by cutoff	Median survival (95% CI)	*P*
Age (y)		18	≥65	11	15 (12-18)	0.5610
			<65	7	13 (10-16)	
KPS		18	90-100	11	14 (11-17)	0.6438
			80	7	18 (8-28)	
Gender		18	M	9	13 (10-16)	0.1937
			F	9	15 (12-18)	
Nestin	First area	17	≥86.415%	9	14 (11-17)	0.1856
			<86.415%	8	19 (12-26)	
	Second area	15	≥13.425%	8	13 (7-19)	0.5001
			<13.425%	7	18 (10-26)	
	Third area	12	≥10.275%	6	13 (11-15)	0.2316
			<10.275%	6	15 (8-22)	
tJNK	First area	14	≥97.520%	7	19 (14-24)	0.1956
			<97.520%	7	14 (11-17)	
	Second area	12	≥99.285%	6	19 (15-23)	0.9779
			<99.285%	6	15 (8-22)	
	Third area	11	≥99.500%	6	19 (17-21)	0.4688
			<99.500%	5	12 (8-16)	
pJNK	First area	14	≥71.565%	7	19 (18-20)	0.2973
			<71.565%	7	13 (10-16)	
	Second area	12	≥48.310%	6	19 (14-24)	0.0564°
			<48.310%	6	12 (6-18)	
	Third area	11	≥32.930%	6	14 (7-21)	0.3497
			<32.930%	5	15 (9-21)	
pJNK/nestin	First area	13	≥0.879	7	19 (17-21)	0.6198
			<0.879	6	14 (8-20)	
	Second area	11	≥2.619	6	19 (16-22)	0.0102*
			<2.619	5	12 (8-16)	
	Third area	10	≥3.496	5	19 (8-30)	0.2178
			<3.496	5	15 (9-21)	
pJNK/tJNK	First area	14	≥0.794	7	19 (17-21)	0.4666
			<0.794	7	14 (11-17)	
	Second area	12	≥0.484	6	19 (14-24)	0.0564°
			<0.484	6	12 (6-18)	
	Third area	12	≥0.448	6	13 (11-15)	0.8361
			<0.448	5	19 (15-23)	
(pJNK/tJNK)/nestin	First area	13	≥0.009	9	18 (9-27)	0.9826
			<0.009	4	13 (0-27)	
	Second area	11	≥0.026	6	19 (16-22)	0.0102*
			<0.026	5	12 (8-16)	
	Third area	10	≥0.035	5	19 (8-30)	0.2178
			<0.035	5	15 (9-21)	

Abbreviation: 95% CI, 95% confidence interval; *statistically significant; °trend toward significance.

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were significantly associated with survival: the median survival time was longer for patients whose pJNK/nestin ratio was ≥2.619 (Fig. 3) and for patients whose (pJNK/tJNK)/nestin ratio was ≥0.026 compared with patients expressing lower values (19 versus 12 months; $P = 0.01$).

Cox proportional hazards model showed that pJNK/nestin and (pJNK/tJNK)/nestin ratios in the second area were independently associated with longer survival time when adjusted for age and KPS ($P = 0.022$ and 0.019, respectively).

Discussion

In this article, we reported for the first time the presence of nestin and pJNK not only in GBM but also in tissue surrounding the enhanced lesion obtained at different distances up to 3.5 cm from the macroscopic tumor margin. Moreover, we highlighted the meaning of pJNK as well as of pJNK/nestin, pJNK/tJNK, and (pJNK/tJNK)/nestin ratios in peritumor areas in terms of survival. In fact, our findings clearly show that, when the values of pJNK and pJNK/tJNK in the second area are ≥48.31% and ≥0.484, respectively, there is a tendency to a longer patient survival with respect to patients expressing lower values (19 versus 12 months for both variables; $P = 0.056$). More importantly, in the second area, when the values of pJNK/nestin and (pJNK/tJNK)/nestin ratios are ≥2.619 and ≥0.026, respectively, the patients' survival is longer (19 versus 12 months for both variables; $P = 0.0102$).

Nestin is expressed by primitive neuroepithelial cells during development and may be considered a marker for neural stem cells in the adult mammalian brain (29). The emerging hypothesis for the origin of brain neoplasias is that they may derive from these stem cells undergoing genetic alterations (4, 12). Nestin is typical of astrocytic tumors and it may distinguish less from more differentiated cells.

In our experience, nestin immunoreactivity is present at high levels in the tumor area in agreement with reports present in the literature (9, 10). Moving to the peritumor tissue, nestin expression sharply decreases. The presence of nestin is due not only to the existence of neoplastic elements, which can be either migrated from the GBM or represent the initiation of independent foci of transformed cells, but also to the existence of reactive glial cells. In addition, nestin immunoreactivity of apparently normal cells may support the idea of an induced premalignant state, which may lead to a full transformation. Nestin immunoreactivity has been found in the endothelium of the tumor vessels and microvessels in agreement with data in the literature (10). In addition, labeled microvessels were present in the peritumor tissue also. This supports the hypothesis that these areas are undergoing an increase in blood supply, which is critical to GBM progression.

The JNK pathway is stimulated by cellular stresses and cytokines (30). Its role in tumorigenesis is conflicting. In fact, JNKs are activated by apoptotic inducers, such as tumor necrosis factor, or high doses of γ radiations (31, 32). Nevertheless, enhanced JNK activation has been found in brain tumor cell lines in response to epidermal growth factor and the duration of activation was greater than that seen for ERK (33). In addition, in GBM, one JNK isoform, JNK2α2, promotes phenotypes associated with tumorigenesis and proliferation (34).

Our findings clearly show that both tJNK and pJNK are expressed in GBM and this agrees with data from other authors (35). In addition, they are present in the endothelial cells of the vessel walls.

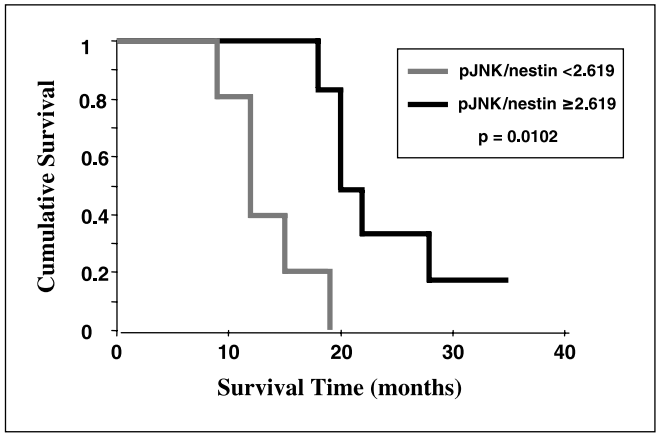

Fig. 3. The Kaplan-Meier plot depicts the differences in survival when patients were dichotomized by the median value of pJNK/nestin ratio. Those patients with pJNK/nestin ratio ≥2.619 in the second area showed a better survival compared with patients with pJNK/nestin ratio <2.619.

tJNK expression is very high both in the tumor and in the peritumor tissue. Nevertheless, a small but statistically significant difference in the expression has been found between the first area and the second and third areas. This agrees with data about tERK1/2 expression reported in our previous article (22) and it is not easy to explain from the biological point of view.

JNK activation is high in GBM but tends to decrease in the peritumor tissue. Nevertheless, no statistically significant difference is seen among the three areas probably due to the great variability of the data and the limited number of observations. The activation in the areas surrounding GBM is seen not only in neoplastic elements when present but in reactive astrocytes and in apparently normal cells also, as observed for nestin expression.

The variability of pJNK expression in the tumor might be explained based on the heterogeneity of cell population. This is supported by data by Antonyak et al. (33), who showed that growth factor stimulation in tumor cell lines derived from GBM induces an activation of JNK different in magnitude and duration depending on cell type. The presence of pJNK in the peritumor tissue may be due to the alteration in the microenvironment created by the tumor mass through the production of various growth factors, inflammatory cytokines, and metabolites released by the necrotic cells. This might not only provoke a variable astrocytic reactivity but also induce in apparently normal cells and in endothelial cells a pJNK expression different in level and distribution. On the other hand, in the investigation in which ERK1/2 expression has been evaluated in GBM and peritumor tissue, the same variability has been observed (22).

In our view, the expression of both nestin and pJNK in the areas surrounding GBM, independently of neoplastic cells, strongly suggests that these areas are changing to a tumor tissue.

If we accept the idea that pJNK may suppress tumor formation by activating apoptosis, of great interest is the relationship in the second area between pJNK/nestin and (pJNK/tJNK)/nestin ratios and survival. It suggests that a better

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prognosis might be linked to the prevalence of the programmed cell death on the presence of tumor stem cells or cells induced to reexpress nestin in association with a transformed status. This condition may be responsible for a resistance to the adopted therapy. Moreover, Korshunov et al. (36) showed in a large cohort of patients a statistically significant association between a high apoptotic index in the tumor and a favorable GBM outcome.

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