FREQUENT CD99 AND FLI-1 EXPRESSIONS IN DIFFUSE LARGE B-CELL LYMPHOMA AND THEIR ASSOCIATION WITH PROLIFERATIVE AND APOPTOTIC RATES

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ABSTRACT

Objective: *CD99 and FLI-1 are best known for their diagnostic utility in Ewing sarcoma/peripheral neuroectodermal tumor (ES/PNET). Recently, CD99 immunoreactivity has been documented in a variety of tumors, including lymphoid malignancies. However, few studies have investigated the FLI-1 immunoreactivity in lymphomas. This study was conducted to determine the frequency of immunohistochemical expressions of CD99 and FLI-1 in diffuse large B-cell lymphoma (DLBCL) samples which may lead to a misdiagnosis. We also sought to determine whether the observed expressions of these markers correlate with tumor cell kinetic parameters such as proliferative index and apoptosis.*

Material and Method: *Using conventional paraffin embedding, immunoperoxidase staining, we retrospectively investigated Ki67 index, CD99 and FLI-1 expressions in 42 DLBCL cases. For in situ detection of apoptosis, terminal deoxynucleotidyl transferase (TdT) -mediated dUTP nick-end labeling (TUNEL) technique was performed. Prechemoterapy primary tumors were used for in all cases study.*

Results: *Immunohistochemical expression of the proteins CD99 and FLI-1 was observed in 14/42 (33.3%) and 7/42 (16.7%) cases, respectively. Concomittant expressions of CD99 and FLI-1 proteins were found in 5/42 (11.9%) cases. The statistical analyses showed a significant positive correlation between Ki67 index and the immunohistochemical expressions of CD99 and FLI-1 (p=0.012 and p=0.046, respectively).*

Conclusion: *This study demonstrates that CD99 and FLI-1 are frequently expressed in DLBCL and in the differential diagnosis of CD99$^+$ and FLI-1$^+$ neoplasms, DLBCL should be considered. The relatively frequent expressions of CD99 and FLI-1 in DLBCL is unexpected, and their biologic and clinical significances have yet to be clarified.*

Key Words: *CD99, FLI-1, lymphoma, diffuse large B-cell*

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DİFFÜZ BÜYÜK B HÜCRELİ LENFOMADA CD99 VE FLI-1 EKSPRESYONU VE PROLİFERATİF İNDEKS VE APOPTOZİS İLE İLİŞKİSİ

ÖZET

Amaç: CD99 ve FLI-1 en iyi Ewing Sarkomu/periferal nöroektodermal tümördeki (ES/PNET) tanısal açıdan kullanımları ile bilinmektedirler. Son zamanlarda, CD99 immünoreaktivitesi lenfoid maligniteleri de içeren farklı tümörlerde bildirilmektedir. Ancak lenfomalarda FLI-1 immüonreaktivitesini araştıran az sayıda çalışma vardır. Bu çalışma diffüz büyük B hücreli lenfoma (DLBCL) örneklerinde yanlış tanıya yol açabilecek CD99 ve FLI-1 ekspresyonunun sıklığını ortaya koymak üzere hazırlanmıştır. Aynı zamanda bu belirteçlerle gözlenen ekspresyonların, proliferatif indeks ve apoptozis gibi tümör hücresi kinetik parametreleri ile korele olup olmadığı araştırılmıştır.

Materyal ve Metod: Konvansiyonel parafin bloklama ve immünoperoksidaz boyama ile 42 DLBCL olgusunda retrospektif olarak Ki67, CD99 ve FLI-1 ekspresyonları araştırıldı. Apoptozisin in situ olarak saptanmasında, terminal deoksinükleotidil transferaz-aracılı dUTP işaretleme (TUNEL) tekniği uygulandı. Tüm olgularda çalışma için kemoterapi öncesi primer tümörler kullanıldı.

Bulgular: 42 DLBCL olgusunun 14 (%33,3) tanesinde CD99 ve 7 (%16,7) tanesinde FLI-1 ekspresyonu saptandı. Olguların 5 (%11,9) tanesinde hem CD99 hem FLI-1 ekspresyonu görüldü. İstatistiksel analizler Ki67 indeksi ile CD99 ve FLI-1'in immünohistokimyasal ekspresyonları arasında anlamlı bir pozitif korelasyon ortaya koydu (sırasıyla p=0,012 ve p=0,046).

Sonuç: Bu çalışma DLBCL'da CD99 ve FLI-1'in sık olarak eksprese edildiğini ve CD99$^+$ ve FLI-1$^+$ neoplazilerin ayırıcı tanısında DLBCL'nın düşünülmesi gerektiğini göstermiştir. CD99 ve FLI-1'in DLBCL'de sık olarak ekspresyonu beklenmedik bir durum olup biyolojik ve klinik olarak önemleri henüz aydınlatılmamıştır.

Anahtar Kelimeler: CD99, FLI-1, lenfoma, difüz büyük B hücreli **Nobel Med 2013; 9(2): 52-56**

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneus disease with different clinical, molecular and biological characteristics.[1,2] The histologic diagnosis of DLBCL usually poses little diagnostic difficulty when appropriate tissue sampling is provided; however, some cases may adopt unusual or unfamiliar appearances mimicking other lymphoproliferative disorders or other malignant neoplasms.[3] Due to the frequent extranodal presentation of the disease and the widely use of core biopsies for diagnostic purposes, the differential diagnosis of DLBCL is broad including epithelial, mesenchymal and other hematologic malignant tumors.

CD99 is a well known membrane epitope for its diagnostic utility in Ewing sarcoma/primitive neuroectodermal tumor (ES/PNET), however its immunoreactivity has been observed in a variety of other tumors including carcinomas, sarcomas, and hematologic neoplasms.[4-6] Thus, Friend Leukemia Integration-1 (FLI-1) has been proposed as an additional immunohistochemical marker of ES/PNET.[7] CD99 expression has been demonstrated in DLBCL and other non-Hodgkin lymphomas in different series, but FLI-1 expression is not well documented.[8-10] Knowledge of the immunoreactivity frequencies and staining patterns of these markers in DLBCL is necessary to avoid misinterpretation of these cases as nonlymphoid malignancies like ES/PNET.

Whether or not CD99 and FLI-1 expressions in DLBCL correlate with tumor cell kinetic parameters such as proliferative rates and apoptosis, remains also unidentified. Increased proliferation and apoptosis are associated with increased tumor grade and aggressive tumor behavior in various B-cell lymphomas including DLBCL.[11] The aims of our study on retrospectively analyzed 42 DLBCL cases were; to investigate the frequencies of CD99 and FLI-1 expressions among our population, and to explore the relationship between these markers and tumor cell kinetic parameters.

MATERIAL and METHOD

Patients: A total of 42 DLBCL patients (20 nodal, 22 extranodal), paraffin-embedded prechemoterapy primary tumor tissues available for immunohistochemical analysis and apoptosis assays were submitted to the analysis. None of the patients had a known history of HIV infection. Pathologically confirmed diagnosis of all cases were made based on recent WHO classification by a senior pathologist (A.H.)[1]. Clinical data were obtained from the hospital records of the GATA Haydarpasa Training Hospital, Istanbul, Turkey. 16 female patients (mean age, 60.4 years; range, 8-87 years) and 26 male patients (mean age, 51 years; range, 20-95 years) were analysed. The protocol was approved by the institutional ethics comittee to permit the use of these samples.

Tissue Macroarray Construction: Tissue macroarray blocks were constructed for 42 DLBCL samples. →

Table 1: Antibodies Used for Immunohistochemistry and Criteria for Positive Staining

Antibody	Clone	Dilution	Manufacturer	Criteria for Positive Immunoreactivity
CD99	H036-1.1	1/40	Cell Marque	Membranous staining in >10% of tumor cells
FLI-1	MRQ-1	1/40	Cell Marque	Nuclear staining in >10% of tumor cells
Ki67	MIB-1	1/75	DAKO	Percentage of tumor cells showing positive nuclear immunoreactivity was calculated as MIB-1 index.

Table 2: CD99 and FLI-1 expression results according to patient demographics, localization, Ki67 index and apoptosis assay

		$CD99^+$ (n=14)	$CD99^-$ (n=28)	$FLI\text{-}1^+$ (n=7)	$FLI\text{-}1^-$ (n=35)	Total (n=42)
Age	Mean (±sd)	58.7 (23.9)	52.5 (21.6)	47.3 (19.5)	56.0 (22.8)	54.6 (22.3)
	Range	8-95	20-90	23-77	8-95	8-95
Gender	M (%)	7 (16.7)	19 (45.2)	5(11.9)	21 (50)	26 (61.9)
	F (%)	7 (16.7)	9 (21.4)	2 (4.8)	14 (33.3)	16 (38.1)
Localization	N (%)	7 (16.7)	13(31.0)	3(7.1)	17(40.5)	20 (47.6)
	EN (%)	7 (16.7)	15 (35.7)	4 (9.5)	18 (42.9)	22 (52.4)
Stage	I-II	7 (17.5)	16 (40.0)	3 (7.5)	20(18.0)	23 (57.5)
	III-IV	7 (17.5)	10 (25.0)	4 (10.0)	13 (32.5)	17 (42.5)
Prolif. rate	Ki67 (±sd)	78.1 (13.1)	64.2 (18.0)	80.1 (11.2)	66.5 (18.0)	68.8 (17.7)
	AI (±sd)	0.16 (0.06)	0.13 (0.05)	0.17 (0.07)	0.13 (0.05)	0.14 (0.06)

Abbreviations: Prolif. Rate: Proliferative rate, M: male, F: female, N: nodal, EN: Extranodal, sd: standart deviation, AI: Apoptotic Index

The selected areas on hematoxylin eosin sections were removed from the paraffin blocks via a 4 mm punch biopsy device. A representative core from each case was transferred to a previously prepared tissue array blocking plate and was re-blocked. Serial 4-µm paraffin sections were cut from the tissue macroarray blocks for apoptosis assays and immunohistochemical studies.

Immunohistochemical Studies and Apoptosis Assays: Immunohistochemical staining with monoclonal CD99 antibody, (CellMarque, Rocklin, CA95677, USA) monoclonal FLI-1 antibody, (CellMarque, Rocklin, CA95677, USA) and monoclonal MIB1 antibody (DAKO, Glostrup, Denmark) were performed according to the standard techniques on a Ventana Benchmark XT autostainer (Ventana Medical Systems Inc., Tucson, USA). A list of antibodies used in this study and criteria for positive immunoreactivity for each antibody are shown in Table 1. Appropriate positive and negative controls were run concurrently for all markers.

Apoptotic cells were labelled by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique, using the In Situ Apoptosis Detection Kit (ApopTag® Plus Peroxidase, In Situ Apoptosis Detection Kit, Chemicon International, Temecula, CA 92590, USA) according to the manufacturer's protocol. Briefly, tissue sections were deparaffinized and rehydrated routinely, then the slides were incubated with proteinase K (20 µg/ml) at room temperature for 15 minutes. After inactivation of endogenous peroxidase by 3% hydrogen peroxide, slides were incubated with equilibration buffer, and then working strength TdT enzymes were applied to sections. After incubation for 60 minutes at 37°C, slides were transferred to a coplin jar containing working strength stop/wash buffer and incubated for 10 minutes at room temperature. Antidigoxigenin conjugate were applied to slides and incubated for 30 minutes at room temperature, then stained with peroxidase substrate, by monitoring the slides under the microscope for optimal staining. Positive control slides given by the manufacturer were used as the positive control specimen for apoptosis, whereas deionized water was used instead of TdT as negative controls. Cells were considered positive when brown reactivity was detected in the nuclei. For apoptotic index assay and Ki67 index, the number of stained cells were counted on three selected areas with the highest number of stained cells and mean value were reported as positive staining cell percentages.

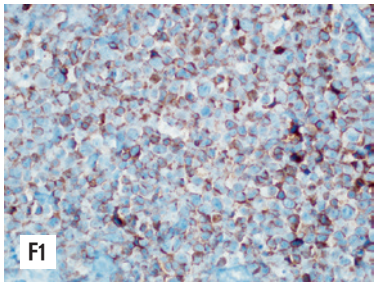
Results of apoptosis assays and immunohistochemical studies were evaluated by two pathologists (U.B. and I.Y.) using a doublehead microscope. Whenever a discrepancy was noted between the observers, a third pathologist (A.H.) was asked to review the cases. The three pathologists reached an agreement on the final scoring.

Statistycal Analysis

Mann Whitney nonparametric tests were used to compare the CD99 and FLI-1 expressions with quantitative parameters of Ki67 index and apoptosis assay results. The association between CD99 and FLI-1 expression was analysed by Spearman's rank correlation coefficient test. The differences of clinical parameters including Ann Arbor stage and presence of B symptoms between the groups were analysed by Chi squared tests. Patients were stratified as having local (stage I-II) or advanced (stage III-IV) disease, and two patients with missing data for clinical stage and presence of B symptoms were excluded in the final analysis. All analyses were performed using SPSS statistical package, version 11.0 for Windows (SPSS, Inc. Chicago, USA). A value of $p<0.05$ was considered as statistically significant. All p values were two-tailed. →

RESULTS

A total of 42 primary tumor samples pathologically diagnosed DLBCL between January 2004 and June 2011 were selected for the analysis. The study population was predominantly male (61.9%) with a mean age of 54.6 years at DLBCL diagnosis. The overview characteristics of patients, results of apoptosis assays and immunohistochemical studies for CD99, FLI-1 and Ki67 are summarized in Table 2. Representative samples of CD99, FLI-1, Ki67 expression and apoptotic assay are shown in Figures 1-4. CD99 and FLI-1 were expressed in 14 (33.3%) and 7 (16.7%) of DLBCLs, respectively. There was a significant positive correlation between CD99 and FLI-1 expressions (Spearman correlation coefficient, r=0.36; p=0.02). The proportion of apoptotic cells as detected by the TUNEL technique were small in all groups, and there were no significant differences among the groups. Mean percentage of Ki67 expression was significantly higher in $CD99^{+}$ group than $CD99^{-}$ group (78.1% and 64.2%, respectively) by Mann-Whitney test (p=0.012). The Ki67 index of FLI-1 positive cases were significantly higher than that of FLI-1 negative cases (80.1% and 66.5%, respectively, p=0.045). Also the group showing concomittant expression for both CD99 and FLI-1 proteins had the highest Ki67 index in all groups (83.4%, p=0.011). FLI-1 expression was not associated with clinical stage (p=0.39) or presence of B symptoms (p=0.69). Despite the difference of clinical stages between $CD99^{+}$ and $CD99^{-}$ patients were not statistically significant (p=0.48), CD99 expression was significantly associated with presence of B symptoms (p=0.015). There were no any significant differences between the age, localization, and gender groups according to the CD99 or FLI-1 expression.



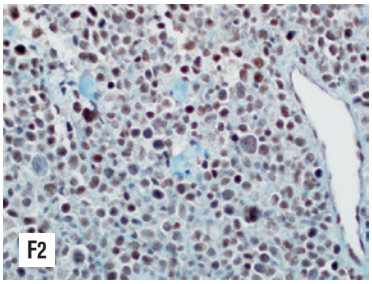
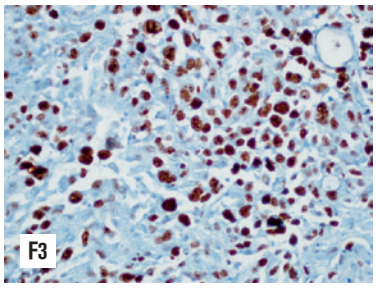


Figure 1: Membraneous CD99 expression in DLBCL (400X)
Figure 2. Nuclear FLI-1 expression in DLBCL (note the immunoreactivity in vascular endothelial cells as internal positive control) (400X)



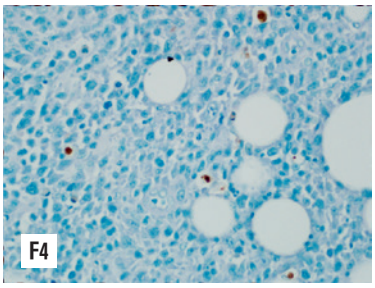


Figure 3. Ki67 immunoreactivity in DLBCL (400X)
Figure 4. Apoptotic tumor cells detected by TUNEL method in DLBCL (400X)

DISCUSSION

Forty two cases (26 men and 16 women) were analysed. Most cases showed the classic morphologic appearance of DLBCL, but cases mimicking Burkitt lymphoma, small cell carcinomas, sarcomas and epithelial malignancies were encountered in this series. The differential diagnosis of DLBCL is broad, difficult to define histologically and can be confused with other neoplastic conditions, especially when appropriate tissue sampling is not provided. Awareness of frequent immunoreactivities of CD99 and FLI-1 in DLBCL is necessary to avoid misinterpretation of these cases as nonlymphoid malignancies like ES/PNET.

With its molecular, clinical and biologically distinct subtypes, DLBCL has been a good model for the study of lymphomas in the last few years. We used immunohistochemical staining for CD99 and FLI-1 to discriminate a subgroup of DLBCL cases by tumor cell kinetic parameters such as apoptosis and Ki67 index, in a study with archival tissues. Establishment of such markers will also facilitate subtyping of this heterogeneous disease in routine clinical care.

Although its function is not well known, Ki67 is expressed in late G1, S, G2, and M phases, and overexpression of Ki67 correlates with growth fraction in all tumor types and associates with poor prognosis in different tumor types.[12] The significant positive correlation between Ki67 index and the immunohistochemical expressions of both markers; CD99 (p=0.012) and FLI-1 (p=0.046) found in the present study is particularly corresponds with the results of a previous report which demonstrated that CD99 expression in DLBCL correlates with poor prognostic factors such as advanced stage at onset, frequent association with poor International Prognostic Index components and non-Germinal Center B Cell subtype.[9] Although our results did not support the association between CD99 expression and advanced clinical stage, we observed a significant association between CD99 expression and presence of B symptoms. Considering these findings together, it appears that CD99 expression seems to be more common in a biologically aggressive substantial part of DLBCL, and the positive correlation of the Ki67 index with FLI-1 and CD99 expression found in this study may be related to unfavourable effects on clinical outcome of the disease. →

The important role of increased cell survival and anti-apoptotic proteins like bcl-2 in lymphomagenesis is evidenced by the accumulation of data over recent years, and the association of specific lymphoma subtypes with specific regulators of apoptosis.[13-15] However, factors that contribute to antiapoptotic mechanisms in most lymphoma subtypes are mainly lacking. Since the normal and aberrant FLI-1 fusion proteins have been shown to inhibit apoptosis, we were also interested in comparing relative numbers of apoptotic cells in DLBCL.[16] However, the numbers of apoptotic cells shown by the TUNEL technique were small in all groups and there were no significant differences among the groups. Mhawech-Fauceglia et al. found FLI-1 expression in 8/32 (25%) of DLBCL samples in their study.[17] Our results confirm the FLI-1 expression in DLBCL with a similar frequency 7/42 (16.7%).

CONCLUSION

Although the present study is limited by its retrospective design with a small number of patients, it documents the unexpected concomittant expressions of CD99 and FLI-1 in DLBCL, which has not been previously reported. Awareness of these immunoreactivities in DLBCL and also in some of other lymphoproliferative disorders will avoid misdiagnosing these cases as ES/PNET. On the other hand, whether CD99 or FLI-1 expression in DLBCL represent clinically distinctive subtyes of the disease remains unanswered, and the potential role of these proteins in the pathogenesis of DLBCL needs to be clarified with further investigations. Therefore, prospective well-designed clinical trials are needed in order to evaluate the prognostic impacts of these proteins in DLBCL cases.

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