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Original Research Article

Molecular Identification of Cancer Stem Cells in Pediatric Solid Tumours and Its Correlation with Clinicopathological Profile

Vasugi Gramani Arumugam[1*], Sandhya Sundaram[2], Latha3, Julius Xavier Scott[4]

[1]Assistant Professor, Department of Pathology, Sri Ramachandra Medical College, No.1, Ramachandra Nagar, Sri Ramachandra Nagar, Porur, Chennai, Tamil Nadu 600116, India
[2]Professor, Department of Pathology, Sri Ramachandra Medical College, No.1, Ramachandra Nagar, Sri Ramachandra Nagar, Porur, Chennai, Tamil Nadu 600116, India
[3]Assistant Professor, Department of Pediatric Oncology, Sri Ramachandra Medical College, No.1, Ramachandra Nagar, Sri Ramachandra Nagar, Porur, Chennai, Tamil Nadu 600116, India
[4]Professor, Department of Pediatric Oncology, Sri Ramachandra Medical College, No.1, Ramachandra Nagar, Sri Ramachandra Nagar, Porur, Chennai, Tamil Nadu 600116, India

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***Corresponding author:** Dr. Gramani Arumugam Vasugi

Abstract

Pediatric solid tumours constitute a unique division of importance not only for the age group affected but also for the increased incidence of recurrence and relapse. OBJECTIVE: The aim of this study is to study the clinicopathological profile of malignant pediatric solid tumours and also to analyze the expression pattern of cancer stem cell marker, CD44 in a series of pediatric solid tumours. ***Materials and Methods:*** 75 cases of malignant solid tumours age group less than 17 years, reported at Sri Ramachandra Medical College from jan 2009 to dec 2013 were included in this study. Formalin fixed paraffin embedded tissue stained with H&E were used for routine morphology. Immunohistochemical staining was done using monoclonal anti CD-44 antibody by Streptavidin biotin peroxidase complex technique. Scoring was done using Histochemical Scoring (H-Score) method. ***Results:*** Among the 75 cases 52 (68%) were males and 23(32%) were females. 22(29%) were CNS neoplasms and 53(71%) were Non CNS neoplasms which included lymphoma, Ewing's sarcoma, Osteosarcoma, Wilms tumour, Soft tissue tumours, Germ cell tumours, Neuroblastoma, Hepatoblastoma & others. The CD 44 expression was variable according to the grades of CNS tumours. Among non CNS neoplasms, lymphomas showed an increased expression for CD44 with all the 11 of 12 cases (91%) being positive [Mean H-score-213.4]. Ewing's sarcoma/PNET (9 cases) and Wilm's tumour (5 cases) were completely negative for CD44. ***Conclusion***: Pediatric solid tumours form a wide spectrum. Targeting CD 44 CSCs could be a strategy to improve the outcome in these tumours.

Keywords: CD-44, Pediatric solid tumours, prognosis, Immunohistochemistry.

INTRODUCTION

Pediatric cancers differ significantly from adult cancers in terms of incidence, etiology, response rate to current therapies, and outcome, mainly due to major differences in tumor biology. A subpopulation of cells, termed tumor-initiating cells or tumor stem cells (TSC), have been identified in many different types of solid tumors. These TSC, which are typically more resistant to chemotherapy and radiation compared to other tumor cells, have properties similar to normal stem cells including multipotency, ability to self-renew, proliferate, and maintain the neoplastic clone. With considerable differences in tumor biology between adult and pediatric cancers, there may be significant differences in the presence, function and behavior of TSC in pediatric malignancies.

The main categories include brain tumours, neuroblastoma and ganglioneuroma, Lymphomas, Ewing's sarcoma/ PNET, Wilm's tumor, rhabdomyosarcoma, germ cell tumor, osteosarcoma, & hepatoblastoma. Pediatric brain tumours accounts for 20% of all pediatric malignancies [1]. The most prevalent primary brain tumours among the pediatric population are astrocytomas, ependymomas, medulloblastomas, glioblastoma multiforme, craniopharyngiomas, choroids plexus neoplasms, etc.

CD44 (also known as homing cell adhesion molecule) is a cell surface glycoprotein expressed on

lymphocytes, monocytes, and granulocytes, and has been identified as a stem cell marker in breast, head and neck, pancreas, and colon cancer. This cell surface glycoprotein is a polymorphic molecule that results from alternative splicing and cell lineage specific glycosylation [2]. The most prevalent isoform of CD 44 is a 80- to 90- kd molecule named CD 44H. CD 44 molecules act as the principal receptor for hyaluronate. Experimental models, both in vitro and in animals provide evidence that overexpression of CD 44H or its variant is correlated with enhanced tumorigenicity and metastatic behaviour.

The study was designed to analyze the histological spectrum of Pediatric solid tumours, also to find out the expression of CD 44 and its correlation with tumour and clinical characteristics. To our knowledge, this is the first immunohistochemical comparative study on a heterogeneous collection of pediatric solid tumors using the reported CSC marker in the Indian context.

MATERIALS AND METHODS

This is a retrospective study which includes 75 cases of pediatric solid tumours, age group between 0 to 17 yrs who presented in SRMC medical center from 2009 to 2013. The records of the patients were retrived from pathology database and analyzed. Formalin fixed 3mm sections were stained by H&E and then by IHC with monoclonal anti CD-44 antibody using streptavidin biotin peroxidase complex.

Initially, slides were scanned at 10×magnification to obtain a general impression of the overall distribution of the tumor cells and positive cells were then assessed semi-quantitatively at higher magnifications and final scores were given. Scoring was done using Histochemical Scoring (H-Score) method.

For CD 44 marker the intensity of staining was scored on a scale of 0 to 3 as, 0 (absent), 1+ (weak), 2+ (moderate), 3+ (strong). The percentage of tumour cells showing positive staining was also assessed semi-quantitatively. In addition, the H-score of immunoreactivity was obtained by multiplying intensity and percentage of positive cells and a final score of 0 to 300 was given. The median of expression was chosen as cut-off value to classify the sample as positive or negative.

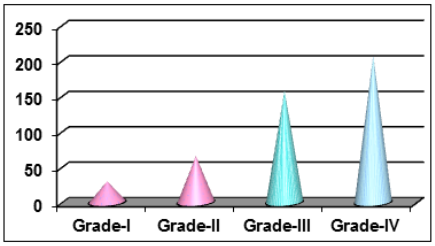
Statistical Analysis

The mean, standard deviation and standard error of mean for CD-44 was calculated in all the pediatric solid tumors. The correlation between various grades of CNS neoplasms with CD-44 was done using Kruskal Wallis one way analysis and Pearson correlation test.

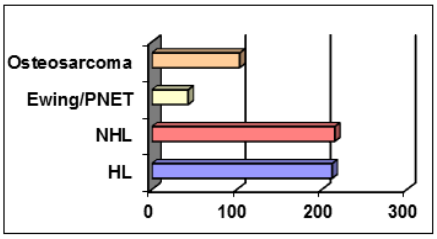
RESULTS

The pattern of CD 44 expression in PST'S was mainly seen in the membrane with no background staining of the stroma or nuclei. The intensity of staining and percentage of positive cells was evaluated in different grades of CNS neoplasms and other pediatric solid tumors and finally analysed using the H-score.

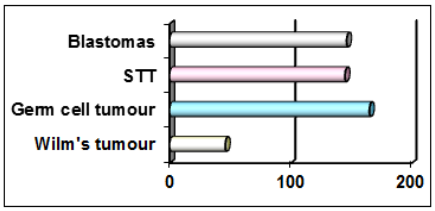
The median value [H score-140] was taken as the cutoff to segregate cases as positive and negative. Eleven (50%) out of 22 cases of grade I and II CNS neoplasms were negative [Mean H score-47.5]. The remaining 11(50%) cases belonging to grade III and IV were positive [Mean H-score-187.2]. The Mean H-score of various grades of CNS neoplasms are expressed in Graph-1. There was a positive correlation between CD 44 with increasing grades of pediatric brain tumours which was statistically significant ($p < 0.05$).



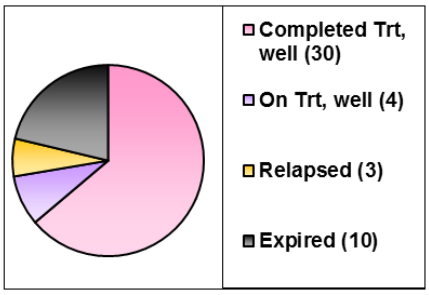
Graph-1: Mean CD 44 Expression in CNS Neoplasms



Graph-2: Mean CD44 in Other Pediatric Solid Tumours



Graph-3: CD44 Expression in Other Pediatric Solid Tumours



Graph-4: Follow Up Data in a Total of 47 Cases

Table-1: CD 44 Expression

TUMOUR	Number of cases	Minimum H-Score	Maximum H-Score	Mean H-Score	Std. Error of mean	Std. deviation
Brain tumours	22	10.00	234.00	124.772	17.185	80.6083
Lymphomas	12	130.00	260.00	212.500	10.179	35.2613
Ewing's/PNET	9	21.00	128.00	42.667	11.007	33.0227
Osteosarcoma	6	25.00	180.00	103.333	21.705	53.1664
Wilms	5	35.00	55.00	46.000	3.7947	8.48528
Germ cell	4	145.00	191.00	173.250	9.9697	19.9394
STT	5	55.00	195.00	144.800	26.480	59.2131

Table-2: Expression of Ki-67 and CD 44 in Various Grades of Pediatric Brain Tumour

	Grade	N	Mean H-Score
Ki-67	I	4	0.5%
	II	7	2.3%
	III	2	18.4%
	IV	9	28.8%
	Total	22	

Kruskal- Wallis one way Analysis of variance
Chi- Square = 17.871, Degrees of freedom = 3, P value = 0.0001

	Grade	N	Mean H-Score
CD 44	I	4	29.7
	II	7	65.5
	III	2	157.5
	IV	9	206.8
	Total	22	

Kruskal- Wallis one way Analysis of variance
Chi- Square = 18.035, Degrees of freedom = 3, P value = 0.0001

Table-3: Correlation between CD 44 and Ki-67 in Various Grades of CNS Neoplasms

		CD 44	Ki-67
CD 44	Pearson Correlation	1	.873**
	Sig. (2-tailed)		.000
	N	22	22
Ki-67	Pearson Correlation	.873**	1
	Sig. (2-tailed)	.000	
	N	22	22
**. Correlation is significant at the 0.01 level (2-tailed).			

Table-4: Variance in CD 44 across Various Grades of Pediatric Brain Tumours

Variable	Grades compared	P value
CD 44	II & III	0.056
	I & IV	0.003
	I & III	0.133
	I & II	0.042
	III & IV	0.036
	II & IV	0.0001

Table-1 shows the mean, standard deviation and standard error of mean values for CD 44 expression in all tumours. The mean values were highest in lymphomas followed by germ cell tumours and CNS neoplasms. The mean values of CD 44 expression and its correlation with various grades of CNS neoplasms are tabulated in Table-2. The statistical workup was analysed using Kruskal Wallis one way analysis which was significant ($p < 0.05$). Table-3 shows the correlation between Ki-67 labelling Index and expression of CD 44 among various grades of CNS tumours which was also statistically significant ($p < 0.01$). The variance between the expression of CD 44 and WHO grades of CNS neoplasms with their statistical significance is tabulated in Table-4.

Among non CNS neoplasms, lymphomas showed an increased expression for CD44 with all the 11 of 12 cases (91%) being positive [Mean H-score-213.4]. Ewing's sarcoma/PNET (9 cases) and Wilm's tumour (5 cases) were completely negative for CD44.

Five out of six cases showed negative expression in osteosarcoma while all four cases of germ cell tumour showed positive staining for CD 44. 3(60%) out of 5 cases of soft tissue tumours were positive. 4 cases of blastomas [Mean H-score-146.2], 2 cases of papliiary carcinoma thyroid [Mean H-score-162.5], one case of malignant melanoma [H-score-215], SCC [H-score-167], Adenoid cystic carcinoma [H-score-235] were positive for the staining. The expression of CD 44 in non CNS tumours are depicted in Graph 2 & 3.

Patients follow up (Available data)

Patient follow up could be collected for 47 cases. Out of the 47 patients, 10 children expired, 13 children were under various cycles of chemotherapy, 12 patients with CNS neoplasms recovered with surgery and radiotherapy and 8 patients have completed the treatment with full recovery and are on followup. Recurrence was found in 4 cases (Graph-4). The children with a diagnosis of Medulloblastoma, Glioblastoma multiforme, High grade sarcoma, Botryoid rhabdomyosarcoma, Wilm's tumour, Osteosarcoma, Neuroblastoma expired in the course of treatment.

DISCUSSION

Despite dramatic improvements in cancer treatment in the past few decades, two of the major challenges are relapse and tumour resistance to therapy. These challenges may result from residual cancer stem cells which may be resistant to conventional chemo- and radiotherapies and are therfore difficult to eradicate.

Gerber *et al*., [3] demonstrated, for the first time, that the presence of CSCs in AML correlates with a poor clinical outcome and suggested that those cells are responsible for tumour relapse. In the present study, we investigated the expression of cancer stem cell

marker CD 44 in pediatric solid tumours presented at our Institute over a period of five years.

The analysis of CD 44 based on H-Score revealed that among the most common PST's 91% of lymphomas, 50% of brain tumours and 100% of germ cell tumors showed a positive expression.Soft tissue tumors and osteosarcomas showed a moderate expression while Ewing's sarcoma and Wilm's tumour showed a negative staining pattern for CD 44.

The mean expression of CD 44 using H-Score for Grade I, II, III, and IV tumours are 29.7, 65.5, 157.5, and 206.8 in order. The correlation between CD 44 expression and grading was found to be statistically significant ($p < 0.05$). All Grade I and II tumours (11/11) were negative and all grade III and IV tumours (11/11) were positive. This was contrary to a study by L R Ylagan, B Quinn *et al*., [4] where there was no significant correlation between the staining patterns of CD 44 in low grade and high gliomas.

Lymphomas, both Hodgkin's and Non Hodgkin's lymphoma had a striking increase in CD 44 expression. The mean H-Score being 212.2 in Hodgkin's and 213.4 in Non Hodgkin's lymphoma. 91% of cases (11/12) showed positivity which correlates with a study by N Tacyildiz, A. O. Cavdar *et al.*, [5].

All cases of germ cell tumours (4/4) were positive for CD 44 staining with a mean H-Score of 173.2. The expression pattern observed in other solid tumours were relatively less. Ewing's sarcoma showed only 11% positivity (1/9) and in Osteosarcoma it was only 16% (1/6). All cases of Wilms tumour (5/5) were negative for CD 44 staining which is in agreement with a study by Mitra Mehrazma, Zahra Madjd *et al.*, [6] were 97% of were reported negative. In soft tissue tumours 3 out of 5 cases were positive and all three cases of neuroblastomas were positive. Among other cases of pediatric solid tumours, malignant melanoma showed a strong positive expression for CD 44.

Similar to other investigated cancers, we found that in PSTs, the number of cancer cells expressing CD 44 was far too high to be limited to a cancer stem cell population, concluding that CD 44 expression alone cannot determine "stemness." A major limitation of this approach is that not all cancer cells expressing a suggested biomarker have functional characteristics of CSCs, and conversely, cancer cells that lose their CSC marker expression may act like CSCs [7, 8].

Currently, literature reports that researchers are trying to identify the specific markers or sets of markers to characterize a pure CSC population [9]. An effective surface marker for targeted therapy should identify all tumor CSCs and must prove to be a unique marker that is not expressed by normal cells.

Recently, Balla *et al.*, reported that CD44, is also involved in a wide range of cell functions including adhesion and migration, & is expressed by retinoblastoma stem-like cells [10]. The implication of CD44 as a cancer stem cell marker has been previously reported for adult cancers, such as breast, head and neck, colon and pancreatic cancers [11-15].

We found a positive association between CD 44 expression with increasing grades of pediatric brain tumours. There was also a marked staining of CD 44 in pediatric lymphomas and germ cell tumours. Other tumours showed a varied expression pattern. In order to better establish cancer stem cells, the stained cells should be isolated from PST tissues by flow cytometry and their in vitro biologic characteristics should be determined.

On reviewing the clinical outcome of these patients, we found that the mortality was high in sarcomas particularly osteosarcomas and in grade IV CNS neoplasms (Medulloblastomas and glioblastoma multiforme). One case of neuroblastoma and lymphoma expired due to relapse of the disease even after completing treatment. In our study, we found a positive correlation between the expression of CD 44 in grade IV CNS neoplasms and the clinical outcome. A cancer research statistics on childhood cancer mortality say that the most common cancer deaths occur in pediatric brain tumours followed by tumours of the sympathetic nervous system [16].

Cancer stem cells has recently been discovered in a variety of pediatric solid tumours and the knowledge of pediatric CSCs identity, their function, microenvironment and resistance pattern is still a gray area. However, in depth multicentric study with larger sample size may be required to determine and delineate the specific cancer stem cell markers [17]. Once there is a greater understanding of pediatric TSC, novel, targeted therapies can be developed to help eradicate these resilient cells, and hopefully improve outcomes for children with difficult to treat or relapsed solid tumours.

CONCLUSION

In, conclusion, our study established the presence of putative stem cell marker, CD 44 predominantly in CNS neoplasms, Lymphomas and Germ cell tumours and its expression correlates with the aggressive nature of the lesion. Targeting CD 44 CSCs could be a strategy to improve the outcome in these tumours. Hence, the analysis of CD 44 expression may be recommended as an additional adjunct in assessing Pediatric solid tumours.

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