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Serum Prohepcidin and Serum Ferritin The Diagnostic And Prognostic Markers in Primary Abdominal Solid Tumours of Paediatric Patients

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***Abstract*— MATERIAL & METHODS- This study conducted in the Department of Paediatric Surgery and the Department of Immunology & Molecular Medicine Sher-I-Kashmir Institute of Medical Sciences Srinagar, is a prospective study. The study comprised of twenty patients with primary abdominal solid tumours between the age group of 2 to 15 years and twenty normal controls between the age group of 2 to 15 years from Kashmir valley. Complete surgical excision of resectable tumours was done, while in unresectable tumour cases, only biopsy was taken & tissue diagnosis was made by histopathology. In all the cases, venous blood samples were taken before and after surgery upon proper consent. We took venous blood samples in all the controls upon proper consent. From the samples, the serum after separation was stored at −20o C until processed. We used only once thawed samples to measure serum prohepcidin and serum ferritin levels according to proper procedures and protocol prescribed in the kit.**

Results; shows correlation of post-operative serum prohepcidin with hematologic and iron parameters and inflammatory markers in solid tumours. The positively correlation between pre-operative serum prohepcidin level with haemoglobin and the negative correlation between post-operative serum prohepcidin level with ESR and post-operative serum ferritin.

Conclusion; serum ferritin and Prohepcidin are important biochemical marker in intra-abdominal tumors and its estimation is helpful in diagnostic and prognostic estimation of inta abdominal tumors but the thing is normal range of prohepcidin estimation which is to be elucidated yet.

I. INTRODUCTION

Cancer is the second leading cause of death in children (after trauma) and childhood cancer is the leading cause of death by disease in children between infancy and age 15[1].Cancer in children and adolescents is relatively rare: although the overall incidence of childhood cancer has been slowly increasing since 1975. More children present with cancer each year in developing countries than in developed nations, and developing countries seem to bear a gradually increasing proportion of worlds' burden of childhood cancer [21]. It is estimated that 1 in 600 children have a chance of developing cancer during the first 14 years of life [21]. Worldwide the annual number of new cases of childhood cancer exceeds 200,000 and more than 80% of these are from the developing countries [1]. Childhood cancer accounts for approximately 11% of all paediatric deaths in United States [9]. The distribution of paediatric malignancies in western countries is leukaemia's (30%), central nervous tumours (20%), lymphomas (10%) & neuroblastomas (8%). The incidence of childhood cancer in most populations of the world ranges from 75-150/million/year and in India; it is 38-124/million/year [1]. Overall cancer is more common in males (39-150/million/year) than in females (23-97/million/year), however Wilma tumour has female preponderance. Leukaemia is most common childhood malignancy [2]. Neuroblastoma is the second most common childhood solid tumour but it is much less frequently reported in India [3]. A study from Kashmir has revealed the distribution of various primary abdominal solid tumours as Wilms tumour 37.5%, neuroblastoma 15%, lymphomas 32.5%, hepatocellular carcinoma 2.5% and others (including teratocarcinoma stomach) 12.5% in the paediatric patients of Kashmir.

Primary abdominal tumours though relatively rare in children, attract considerable notice because of the possibility of malignant disease, serious prognosis, high cost of treatment and emotional & psychological trauma to the patients and their parents (4). A palpable mass is the most common sign suggesting diagnosis of a malignant tumour in a child. However, as reported by Melicow & Uson, 45% of abdominal masses do not have a malignant cause. It is imperative that a child be referred quickly to the appropriate specialist as initial & proper evaluation is of great value in deciding the appropriate management [21]. Thus, early diagnosis & proper management of abdominal solid tumours in children is of utmost need. Earlier, management of these tumours had numerous limitations. Advances in diagnostic methods and the application of vigorous multidisciplinary treatment polices have resulted in marked improvement in treatment of primary abdominal solid tumours of children because if diagnosed earlier, these tumours respond better to treatment, resulting in better survival & prognosis (5). The evaluation of a child with an abdominal mass involves a number of diagnostic considerations. The diagnostic modality utilized depends on age and gender of the patient, the location of the mass, and the presence or absence of other related signs and symptoms, as well as features on general physical examination (6). Despite all the investigation modalities available presently, their use in children is of great concern because the diagnosis of most of the abdominal tumours involve procedures like fine needle aspiration cytology/fine needle aspiration biopsy, bone marrow biopsy, ultrasonography (USG) or computed tomography (CT) guided biopsy, magnetic resonance imaging (MRI) which are

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either invasive/costly or both (7). A clinical study of primary abdominal tumours in children of Kashmir conducted in Sher-i-Kashmir Institute of Medical Sciences Srinagar, in the Department of Paediatric Surgery and the Department of Medical Oncology revealed that there was unawareness in general public about these abdominal masses and there were lack of techniques for the early assessment of these tumours. However, the disease has been observed to be relatively common in the region of South-Kashmir & anaemia found to a predominant feature in these patients. While, most patients presented to the tertiary care centre at a late stage, many of them had been treated as cases of worm colic, worm masses.

Despite successes in treating solid tumours such as Wilms tumour, disappointments in the outcomes of high-risk solid tumours like neuroblastoma have precipitated efforts towards the early and accurate detection of these malignancies (8). Survival rates for children with cancer have improved over the past decades. Recent trends show that although survival rates for children with primarily leukaemia's and lymphomas have improved, five-year survival rates for paediatric solid tumours have not changed much over the past two decades (10). The limited numbers of clinical trials on paediatric solid malignancies as well as difficulty in discriminating and diagnosing solid masses by using standard techniques have hindered progress in this area. Therefore, it is imperative to not only accurately determine the type of masses but also detect tumour recurrences. Determination of the organ or tissue of origin of the mass can narrow down the diagnostic possibilities considerably. However, improved survival of children with these tumours awaits better detection, chemotherapy and understanding of the molecular basis of the growth of these tumours [11]. Serum tumour markers offer great promise in the management of children with solid malignancies and are integral to disease diagnosis and prediction of treatment response. These cancer biomarkers are biologically measured substances that are expressed by malignant tissues, circulating tumour components, or generated by the host in response to the tumour, and constitutively serve as essential tools to aid clinicians in making diagnosis, staging, and risk assessments (12). The nonspecific property of some markers to be differentially expressed in other tissues limits their clinical use and hampers accurate diagnosis of disease information. Despite considerable potential limitations to current paediatric tumour markers, continued progress in tumour marker discovery will likely come from investigations that will integrate multidimensional analysis of more specific predictive and prognostic markers (13). Next-generation biomarkers will likely stem from the growing technologies, to promote personalized cancer care. Moreover, there is a growing opinion in favour of the role of targeting proteomic measurements for improving blood-based human diagnostics (14). Blood has been the logical biomarker source, as blood equilibrates with tissues and generally harbours proteins that may be identified to yield a specific proteome pattern related to a distinct pathologic process occurring in the body (15). To date, blood-based proteomic biomarker efforts have had little success, in large part because the relatively small number of highly abundant proteins make the reliable detection of low abundant disease-related proteins challenging due to the wide dynamic range of concentrations of proteins in a blood sample. The progress towards early diagnosis, curative therapy, and favourable outcomes in childhood solid cancer patients and survivors depends on identifying biomarkers having diagnostic and prognostic role (16). Given the paucity of data on biomarkers and the challenging clinical management of children with solid tumours, a cross-disciplinary biological approach that comprehensively assesses DNA, RNA, protein, and metabolites to identify molecular drivers and markers is warranted to conduct small, short, and more economical individualized clinical trials (17).

Serum biomarkers are useful tools to differentiate solid tumours in children. There is an abundance of literature on serum biomarkers. Although these laboratory tests are helpful in discriminating masses, some markers do not always accurately indicate disease, but are in general reliable when used in combination with other diagnostic tools. Serum biomarkers may be required for planning future cancer treatment (18).

Ferritin levels in serum have been used to evaluate clinical conditions not related to iron storage, including inflammation, chronic liver disease, and malignancy. One of the known tumour marker of neuroblastoma is serum ferritin. Hepcidin is a low-molecular-weight hepatic peptide that regulates iron homeostasis, and acts by causing the degradation of its receptor, the cellular iron exporter ferroportin (19). Based on the major role of the hepcidin-ferroportin axis in iron regulation, recently several studies have discussed its expression and influence on the development and prognosis of cancer. Iron plays a pivotal role in homeostasis. However, insights into the mechanisms of normal iron regulation have provided a new perspective on the basic mechanisms, biological rationale, and pathophysiologic implications of changes in iron metabolism in cancer (20). Besides being a crucial stimulus for cancer, iron dysfunction also causes cancer-related anaemia. Significant positive correlation of serum prohepcidin with serum ferritin level and significant negative correlation of serum prohepcidin with haemoglobin in children with solid tumours has been found. Elevated Serum prohepcidin may be clinically important predictor of inflammation and leads to anaemia by impairing iron utilization in solid tumours (21). Studies in adults with cancer have shown a significant correlation with serum prohepcidin. Because hepcidin can convey important information about pathologic states, the ability to measure hepcidin in either plasma or urine has considerable clinical application [19, 6]. Higher serum prohepcidin levels have been found in patients with renal tumour that has been associated with the extent of disease and unfavourable clinical outcome. A few studies worldwide have examined the relation of prohepcidin levels with solid tumours in children. These studies indicate that serum prohepcidin level was elevated in children with solid tumours and could be more indicative of failure of erythropoiesis rather than iron deficiency (22). A recent study reported from Turkey (Zühre Kaya et al., 2010) has related the serum prohepcidin levels in children with solid tumours; it has been reported that serum prohepcidin was significantly higher in solid tumours than in iron deficiency anaemia and healthy controls. Worldwide, assessment of the prognostic and diagnostic potential of serum prohepcidin in various

primary abdominal solid tumours is under evaluation. Besides being non-invasive, the estimation of serum prohepcidin levels is highly sensitive, economical and provides early results. Thus, the present study aims to evaluate the serum prohepcidin and ferritin levels that can aid in the early diagnosis & management of the paediatric patients with primary abdominal solid tumours (23).

II. MATERIAL & METHODS

This study conducted in the Department of Paediatric Surgery and the Department of Immunology & Molecular Medicine Sher-i-Kashmir Institute of Medical Sciences Srinagar, is a prospective study. The study comprised of twenty patients with primary abdominal solid tumours between the age group of 2 to 15 years and twenty normal controls between the age group of 2 to 15 years from Kashmir valley. We included patients with abdominal solid mass admitted in the Department of Paediatric surgery, Sher-i-Kashmir Institute of Medical Sciences Srinagar in our study. We documented complete history and routine investigations of each case on a proper proforma. We excluded patients with signs of infection or inflammation from the present study. Complete surgical excision of resectable tumours was done, while in unresectable tumour cases, only biopsy was taken & tissue diagnosis was made by histopathology. In all the cases, venous blood samples were taken before and after surgery upon proper consent. We took venous blood samples in all the controls upon proper consent. From the samples, the serum after separation was stored at −20o C until processed. We used only once thawed samples to measure serum prohepcidin according to proper procedures and protocol prescribed in the kit. In all the cases and controls serum prohepcidin levels were measured using human prohepcidin ELISA kit. We used DRG (DRG Instruments, Marburg, Germany) hepcidin prohormone ELISA (a commercially available kit) to measure the serum prohepcidin concentrations according to the manufacturer's instructions. The range of assay is between 0-1000ng/mL. The sensitivity of this assay was < 3.95 ng/ml.

III. PRINCIPLE OF THE PROHEPCIDIN TEST

The DRG Hepcidin prohormone ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the principle of competitive binding. The micro-titer wells are coated with a polyclonal antibody directed towards an antigenic site on the hepcidin prohormone molecule (28). Endogenous hepcidin prohormone of a patient sample competes with a hepcidin prohormone-biotin conjugate for binding to the coated antibody. After incubation, the unbound conjugate is washed off. The amount of bound biotin conjugate is reverse proportional to the concentration of hepcidin prohormone in the sample. After addition of the substrate solution, the intensity of colour developed is reverse proportional to the concentration of hepcidin prohormone in the patient sample.

IV. RESULTS

The present study comprises of the demographic profile (age, weight and gender ratio) of cases and controls illustrated in Table-1. We found significant difference in age between cases and controls (p=0.002). Cases and controls were comparable with respect to weight and gender.

TABLES

Table 1: Comparison of demographic data of solid tumours and controls.

	Solid tumours 20	Controls 20	p value
Age (in years)	4.7 ± 3.55	8.5 ± 3.53	0.002
Weight (in kgs)	16.85 ± 8.27	19.4 ± 8.29	0.336
Gender (male/female)	8/12	12/8	

The table-2 and shows the comparison of laboratory parameters haemoglobin, ESR, serum ferritin and serum prohepcidin among cases and controls. Figures-4, 5, 6 & 7 respectively show comparison between cases and controls with respect to haemoglobin, ESR, serum ferritin and serum prohepcidin. On comparison of these variables between cases and controls, statistically significant differences were found with respect to haemoglobin, ESR, serum ferritin and serum prohepcidin with p value ≤0.0001, ≤0.0001, 0.016 and 0.002 respectively.

Table 2: Comparison of laboratory data of solid tumours and controls.

	Solid tumours 20	Controls 20	p value
Haemoglobin (g/dl)	10.55 ± 2.012	12.4 ± 0.598	<0.0001 (Sig)
ESR (mm/1st hr)	17.2 ± 7.346	6 ± 2.406	<0.0001 (Sig)
Ferritin (ng/ml)	183 ± 176.447	72.55 ± 84.038	0.016 (Sig)
Prohepcidin (ng/ml)	227.5 ± 221.604	58.4 ± 49.381	0.002 (Sig)

The table-3 the comparison of pre-operative and post-operative serum ferritin level in cases. On comparison

between cases and controls, no statistically significant difference was found with p value 0.105.

Table 3**:** Comparison of pre-operative & post-operative ferritin levels in cases.

	Mean	N	Std. Deviation	p value
Pre-op Sr.ferritin(ng/ml)	185.33	18	186.312	0.105 (NS)
Post op Sr.ferritin(ng/ml)	112.83	18	85.175	

The table-4shows the comparison of pre-operative and post-operative serum prohepcidin level in cases. On comparison of these variables between cases and controls, statistically significant difference was found with p value <0.018.

Table 4: Comparison of pre-operative & post-operative prohepcidin levels in cases.

	Mean	N	Std. Deviation	p value
Pre-op Sr.prohepcidin(ng/ml)	233.83	18	233.192	0.018 (Sig)
Post op Sr.prohepcidin(ng/ml)	76.39	18	109.754	

Table-5 shows correlation of pre- operative serum prohepcidin with hematologic and iron parameters and inflammatory markers in solid tumours. The positively correlation between pre-operative serum prohepcidin level with haemoglobin (r = 0.388; p = 0.091) and pre-operative serum ferritin (r = 0.347; p = 0.134) figure-10 and the negative correlation between pre-operative serum prohepcidin level with ESR (r = −0.2158; p = 0.362) was found to be statistically non-significant.

Table 5: Correlation of pre-operative prohepcidin with hematologic and iron parameters and inflammatory markers in solid tumours.

	r	p
Haemoglobin (g/dl)	0.388	0.091
ESR (mm/1st hr)	-0.215	0.362
Pre-op ferritin (ng/ml)	0.347	0.134

The table-6 shows correlation of post-operative serum prohepcidin with hematologic and iron parameters and inflammatory markers in solid tumours. The positively correlation between pre-operative serum prohepcidin level with haemoglobin (r = 0.181; p = 0.472) and the negative correlation between post-operative serum prohepcidin level with ESR (r = −0.120; p = 0.634) and post-operative serum ferritin (r = −0.168; p = 0.505) figure-11 was found to be statistically non-significant.

Table 6: Correlation of post-operative prohepcidin with haematological and iron parameters and inflammatory markers in solid tumours.

	r	p
Haemoglobin (g/dl)	0.181	0.472
ESR (mm/1st hr)	-0.120	0.634
Post-op ferritin (ng/ml)	-0.168	0.505

The table-7 shows serum ferritin and serum prohepcidin in controls with respect to gender. On comparison, no statistically significant difference in serum ferritin and serum prohepcidin in male and female controls was found.

Table 7: Serum ferritin and serum prohepcidin in controls

	Gender	N	Mean	Std. Deviation	p value
Sr. ferritin (ng/ml)	Male	12	70.25	90.318	0.886 (NS)
	Female	8	76.00	79.549	
Sr. prohepcidin (ng/ml)	Male	12	58.67	56.082	0.977 (NS)
	Female	8	58.00	40.939	

The table-8 shows the comparison of demographic data (age, weight and gender ratio) and laboratory profile (haemoglobin, ESR, serum ferritin, serum prohepcidin) between the anaemic and non-anaemic cases. On comparison of these variables between anaemic and non-anaemic cases, statistically significant differences were found with respect to haemoglobin ($p \leq 0.0001$) and ESR ($p = 0.005$). No statistically significant differences were found (p value >0.05) between anaemic and non-anaemic cases with respect to age, weight, sex, ferritin and prohepcidin.

Table 8: Comparison of the laboratory tests between the anaemic and non-anaemic patients with abdominal solid tumours.

		N	Mean	Std. Deviation	p value
Age (in years)	Anaemic	14	4.36	3.835	0.525 (NS)
	Non-anaemic	6	5.50	2.950	
Weight in Kgs	Anaemic	14	16.00	8.691	0.498 (NS)
	Non-anaemic	6	18.83	7.548	
Haemoglobin(g/dl)	Anaemic	14	9.64	1.550	≤0.0001 (Sig)
	Non-anaemic	6	12.67	1.211	
ESR(mm/1st hr)	Anaemic	14	20.00	6.737	0.005 (Sig)
	Non-anaemic	6	10.67	3.724	
Pre-op ferritin(ng/ml)	Anaemic	14	207.64	197.495	0.354 (NS)
	Non-anaemic	6	125.50	105.962	
Pre-op prohepcidin(ng/ml)	Anaemic	14	173.79	163.846	0.099 (NS)
	Non-anaemic	6	352.83	299.808	
Post op ferritin(ng/ml)	Anaemic	13	102.54	81.046	0.425 (NS)
	Non-anaemic	5	139.60	99.435	
Post op prohepcidin(ng/ml)	Anaemic	13	53.77	42.701	0.165 (NS)
	Non-anaemic	5	135.20	199.346	

Table-9 shows gender distribution in anaemic and non-anaemic cases. 62.5% male & 75% female cases were anaemic with haemoglobin < 11.5g/dl.

Table 9: Gender distribution in anaemic and non-anaemic cases.

		Cases		Total
		Males	Females	
Anaemic	Count	5	9	14
	%	62.5	75	70
Non-anaemic	Count	3	3	6
	%	37.5	25	30
Total	Count	8	12	20
	%	100	100	100

Table-10 shows no significant difference in male and female cases with respect to pre-operative (p=0.573) and post-operative (p= 0.586) serum prohepcidin level.

Table 10: Pre-operative and post-operative serum prohepcidin level in male and female cases.

	Gender	N	Mean	Std. Deviation	p value
Pre-op Sr. prohepcidin(ng/ml)	Male	8	267.50	293.119	0.573 (NS)
	Female	12	200.83	167.954	
Post op Sr. prohepcidin(ng/ml)	Male	7	61.29	34.731	0.586 (NS)
	Female	11	86.00	139.617	

Table-11 shows serum prohepcidin level in different abdominal tumour cases. The mean pre-operative serum prohepcidin level was 403.00ng/dl in neuroblastoma, 185.56ng/dl wilms tumour, 93.00ng/dl hepatoblastoma and 299.17ng/dl in rest of tumors.

Table 11: Serum ferritin and serum prohepcidin level in different abdominal tumour cases.

	Pre-op ferritin (ng/ml)	Post-op ferritin (ng/ml)	Pre-op prohepcidin (ng/ml)	Post-op prohepcidin (ng/ml)
Neuroblastoma	484.50±434.87	255.00	403.00±366.28	35.00
Wilms tumour	176.33±143.33	107.89±73.75	185.56±136.65	56.78±43.98
Hepatoblastoma	103.67±44.01	20.00±4.24	93.00±90.07	82.50±62.93
Others	132.17±59.32	127.50±90.37	299.17±306.84	110.67±186.03

Table-12 show gender distribution in different abdominal solid tumour cases. Out of 20 cases, 12 were males and 8 were females. We had 1 male & 1 female neuroblastoma, 6 male & 3 female wilms tumour, 1 male & 2 female hepatoblastoma and 4 male & 2 female other tumour cases.

Table 12: Gender distribution in different abdominal tumours.

	Neuroblastoma	Wilms Tumour	Hepatoblastoma	Others
Male cases	1	6	1	4
Female cases	1	3	2	2
Total	2	9	3	6

Table-13 shows serum ferritin level in different abdominal tumour cases. The mean pre-operative serum ferritin level was 484.50ng/dl neuroblastoma, 176.33ng/dl wilms tumour, 103.67ng/dl hepatoblastoma and 132.17ng/dl in rest of tumours. The mean post-operative serum ferritin level was in 107.89ng/dl wilms tumour, 20.0ng/dl hepatoblastoma and 127.50ng/dl in rest of tumors. We had only two neuroblastoma one of them was un-resectable.

V. DISCUSSION

The anemia observed in cancer patients is complex and may result from a combination of causes; iron deficiency, inflammation, toxicity related to therapy, marrow infiltration, dyserythropoiesis and others (9). Hepcidin, a peptide reducing iron availability for erythropoiesis is induced by inflammation, and elevated hepcidin levels may underlie functional iron deficiency in cancer patients. It has been suggested that Hepcidin level is appropriate marker of anaemia of inflammation (24).

Prohepcidin is one of the regulators of iron metabolism. Few studies examined its relation with solid tumours in children. Prohepcidin measurement might turn to be a useful tool in the diagnosis of paediatric tumours in the near future. It is required to establish a reference interval for this hormone and to investigate important aspects of hepcidin biology i.e. age, sex, and race related differences (25). Our present study examined the "Diagnostic & Prognostic Role of serum prohepcidin and serum ferritin in paediatric patients of Kashmir with primary abdominal solid tumours". The present study examined the changes in serum prohepcidin and serum ferritin concentration in children with solid abdominal tumours and compared them with postoperative levels in normal children. Measurement of hepcidin in biological fluids is a rapidly evolving field, with efforts taken to overcome inherent technical difficulties. Such difficulties have hampered appropriate studies in human patients and in the absence of a readily available serum hepcidin assay, a commercial assay to measure hepcidin prohormone, prohepcidin(DRG ELISA), instead of hepcidin has been used (26,27).

Both groups of our study (patients and healthy controls) matched for age and sex. The results of our study revealed significantly higher serum prohepcidin and serum ferritin values in patients compared to the controls (p<0.05). We also found statistically significant higher levels of ESR in our patients compared to the controls. We observed statistically

significant lower haemoglobin values in cases as compared to controls.

We have not found any significant differences in serum prohepcidin levels in males and females. Our study revealed that in healthy children of both sexes mean serum prohepcidin levels was 58.40ng/dl. Differences in serum prohepcidin concentrations between healthy male and females were observed by Lukkonen et al. in 2006. They reported mean serum prohepcidin concentrations of 254 ng/ml and 227 ng/ml, respectively, for 16 males and 37 females [28]. However, the difference was not statistically significant. Our results confirm Lukkonen's observations. Our study in children with solid tumors revealed non-significant difference in serum prohepcidin level (p value 0.099) with mean 173.79ng/dl in anaemic and 352.83ng/dl in non-anaemic patients. Our study contradicts Zuhr kaya and collegues' (2011), (29) study in children with solid tumors which revealed significant difference in serum prohepcidin level (p value 0.013) with mean 756.1ng/dl in anaemic and 503.4ng/dl in non-anaemic patients. This may be because of the large between-subject variation.

Conclusion; thus we concluded from our study that prohepcidin and serum ferritin is an important biochemical marker in intra-abdominal tumors and its estimation is helpful in diagnostic and prognostic estimation of inta abdominal tumors.

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