

Original Research Article

Clinical significance of vanillylmandelic acid in neuroblastoma

Geeta Rathnakumar*, Smita Bolkar, Kinjalka Ghosh, Nitin Inamdar

Department of Biochemistry, Tata Memorial Hospital Parel east, Mumbai, Maharashtra, India

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*Correspondence:

Dr. Geeta Rathnakumar,

E-mail: Rathnakumarg@tmc.gov.in

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ABSTRACT

Background: The second most common solid tumor in children is Neuroblastoma (NB). In about 90% of cases of NB, elevated levels of catecholamines or its metabolites are found in the urine or blood which includes Vanillylmandelic Acid (VMA) and Homovanillic Acid (HVA). Ferritin, Neuron-Specific Enolase (NSE) and Lactate Dehydrogenase (LDH) are commonly assessed in children suspected to have NB, and the levels of these markers are commonly used for differential diagnosis. Multiple clinical and imaging tests are needed for accurate patient assessment. Iodine 123(123I) Metaiodobenzylguanidine (MIBG) is the first-line functional imaging agent used in neuroblastoma imaging. To evaluate the utility of these marker present study was undertaken with 91 NB patients and 40 normal healthy control.

Methods: The study comprised of blood samples and 24 hour's urine sample from 40 normal healthy subjects and 91 untreated patients with histologically proven Stage III and IV NB cases referred to our institute. Method used for NSE was Enzyme Immunoassay (Elisa), serum Ferritin was MIA, LDH-photometry and VMA by Column Chromatography.

Results: Amongst the parameters studied VMA showed highest sensitivity (91%), specificity (94.4%) positive predictive value (97.8%) and 85% negative predictive value at the cut off levels of 7mg/ ml of creatinine as compared to other studied parameters.

Conclusions: This study suggests that the detection of VMA in combination with routine histological examination, MIBG scan, serum NSE and LDH may improve the diagnosis of Neuroblastoma.

Keywords: Homovanillic acid, Lactate dehydrogenase, Metaiodobenzylguanidine, Neuroblastoma, Neuron-specific enolase, Vanillylmandelic acid

INTRODUCTION

Neuroblastomas (NB) are cancers that start in early nerve cells (called neuroblasts) of the sympathetic nervous system. NB most commonly arises in and around the adrenal glands, which have similar origins to nerve cells. Most adrenal gland tumors are benign. They usually do not cause symptoms and may not require treatment. Malignant adrenal gland cancers are uncommon. Types of adrenal gland, tumors include

- Adrenocortical carcinoma - cancer in the outer part of the gland

- Neuroblastoma, a type of childhood cancer
- Pheochromocytoma - a rare tumor that is usually benign

NB is the second most common extra cranial solid tumor in childhood affecting about 7% of all children with cancer. Over one-half of the patients present with metastatic disease at diagnosis. The average age of children when they are diagnosed is about 1 to 2 years. Nearly 90% of cases are diagnosed by age 5. NB is very rare in people over the age of 10 years. The overall survival rate at 10 years is 55%. The prognosis is age and stage dependent, and there is a significant correlation

between age and stage at diagnosis.¹ NB is often present at birth but is most often diagnosed much later when the child begins to show symptoms of the disease. By the time NB is diagnosed, the cancer has usually metastasized, most often to the lymph nodes, bones, bone marrow, liver, and skin. A condition known as "opsoclonus-myoclonus syndrome" can sometimes be a symptom of NB.

Diagnosis of NB can be complicated. It has been called the "great masquerader" because its symptoms mimic so many other diseases. Even a biopsy might reveal cells that can resemble other small round blue tumor cells, like lymphomas and rhabdomyosarcomas. Only a pathologist familiar with NB can distinguish it.

MIBG uptake is seen in 90% of neuroblastomas, identifying both the primary tumor and sites of metastatic disease.² An MIBG scan is an imaging test. It uses the radiopharmaceutical Metaiodobenzylguanidine (MIBG) to find the neuroblastoma tumor and to see if the disease has spread to other tissues or bones. MIBG is an analog of norepinephrine that was developed in the late 1970s.³ The development of MIBG came from efforts by Wieland et al, to produce an agent that could be used to image adrenal medullary tissue and related tumors such as pheochromocytomas. About 10% of children with neuroblastoma have tumours that don't take up MIBG, which means they can't be found with an MIBG scan. These tumors are called MIBG negative.⁴

In about 90% of cases, elevated levels of catecholamines or its metabolites are found in the urine or blood. Catecholamines and its metabolites include dopamine, HVA, and/or VMA.⁵

Prognostic parameters

Besides histology, there are other factors that are important when determining prognostic significance. Of course, the age at diagnosis is important, with children under the age of 1 year having the best overall prognosis. Different plasma and urinary parameters have been tested as valuable prognostic markers for children with NB, but conclusive results from multivariate analyses are still lacking.

Ferritin, NSE, LDH, and the catecholamine metabolites, VMA and HVA, are commonly assessed in children suspected to have NB, and the levels of these markers are commonly used for differential diagnosis.^{6,7}

Serum markers

Serum (blood) levels of certain substances can be used to help predict prognosis.

NB cells release ferritin, a chemical that is an important part of the body's normal iron metabolism, into the blood. Patients with high ferritin levels tend to have a worse prognosis.

NSE and LDH are made by some types of normal cells as well as by NB cells. Increased levels of NSE and LDH in the blood are often linked with a worse outlook in children with NB.⁸

NSE

NSE may be of interest for the prognostic evaluation and follow-up surveillance in patients with NB. Enolase is a glycolytic enzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate. In patients with suspected carcinoid, islet cell tumor, or NB, who have no clear elevations in the primary tumor markers used to diagnose these conditions, an elevated serum NSE level supports the clinical suspicion.⁸

VMA

Catecholamines are produced by a part of the adrenal gland, called the medulla. The majority (not all) of neuroblastoma's produce the catecholamine metabolites, VMA and HVA. So, testing the urine is very often done to detect disease. It's also used as an indicator for response to treatment. For example, a child newly diagnosed with neuroblastoma with higher than normal levels (as these metabolites are normally found in the urine, anyway) of VMA, would be expected to see the levels drop back towards normal values as s/he begins to respond to therapy. About 5-10 percent of neuroblastoma's do not produce elevated VMA and HVA.

LDH (lactate dehydrogenase)

LDH is another prognostic variable used for NB. High levels of LDH in the blood point to acute or chronic cell damage, but additional tests are necessary to discover its cause. Abnormally low LDH levels only rarely occur and usually aren't considered harmful. It's a frequently done test with NB because increased levels can indicate the presence of cancer or certain other disease.⁹

Ferritin

Ferritin is a universal intracellular protein that stores iron and releases it in a controlled fashion. Elevated serum ferritin levels without a corresponding increase in tissue iron storage have been observed in patients with certain cancers. Serum ferritin is known to be one of the tumor markers for NB. Serum ferritin is elevated in most children with NB who are in stages III or IV, but it is not elevated in those in stage I or II. It has also been observed that iron load caused by blood transfusion shows a greater effect on serum ferritin levels than tumor activity due to NB. The authors propose that the pre-transfusion and post-transfusion serum ferritin values be in comparison with other tumor markers such as urinary VMA, urinary HVA, and neuron specific enolase. The serum ferritin level should not be used as a sole indicator of tumor activity in NB.¹⁰

Aim of the study was to investigate the independent diagnostic value of different markers commonly evaluated in NB patients such as urinary VMA, serum NSE, Ferritin and LDH and to evaluate the utility of these in Indian population.

METHODS

Statistically 40 normal healthy subjects (Group I) and 91 untreated patients (Group II) were selected for the study. Age group was from 1 year to 8 years.

Inclusion criteria

- Patients with histologically proven Stage III and IV NB were included for the study.

Exclusion criteria

- Stage I and Stage II were excluded from the study.

Study period was January to December 2018.

Study population

Group I - 40 normal healthy subjects (Age group was from 1 year to 8 years)

Group II - 91 untreated patients (Age group was from 1 year to 8 years)

These cases were referred to Tata Memorial Hospital, Mumbai for investigation and management of disease. The study comprised of 5.0 ml blood samples and 24 hours urine sample from these cases.

Table 1: Methodology used for different parameters.

Investigations	Analysers/ Method
Serum Neuron-specific enolase (NSE)	Enzyme Immunoassay/ Elisa (CanAg's NSE-EIA Kit Method)
Serum Ferritin	AxSYM Immunoassay analyser/ MIA
Serum Lactate dehydrogenase (LDH)	Hitachi 717/ Photometry
Urinary Vanillylmandelic acid (VMA)	Column method (ion-exchange chromatography)

NSE: (Enzyme Immunoassay)

- NSE is a glycolytic enzyme that catalyzes the conversion of 2-phospho-glycerate to phospho-enol-pyruvate.
- The Can Ag NSE-EIA is a solid-phase, non-competitive assay based on two monoclonal antibodies (derived from mice) directed against two separate antigenic determinations of the NSE molecule. The monoclonal antibodies (MAb) used

bind to the γ -subunit of the enzyme and thereby detects both the $\gamma\gamma$ and the $\alpha\gamma$ form.

- The CanAg NSE-EIA standard curve was plotted absorbance against NSE calibrator concentration. The unknown NSE concentrations can be read from the calibration curve using the mean absorbance value of each specimen.

Ferritin: (Microparticle Enzyme Immunoassay (MEIA) technology)

- Microparticles coated with capture Ab are mixed with sample. Analyte binds to capture antibody.
- Mixture is applied to a glass fiber matrix. Microparticles with bound analyte are captured on the top of the matrix, while the remaining sample is allowed to flow through by washing.
- Enzyme labelled Ab (conjugate) added; binds to bound analyte.
- Substrate added; enzyme (alkaline phosphatase) cleaves a phosphate group from the substrate (4-methyl-umbelliferyl phosphate or MUP) to produce a fluorescent product (MU).
- Fluorescent signal is measured. Amount is directly proportional to analyte concentration.

LDH: (Photometry)

- LDH catalyses the oxidation of lactate to pyruvate coupled with the reduction of NAD⁺ to NADH.
- The increase of NADH is measured at 340 nm and is directly proportional to the LDH activity in the sample.

VMA: (Column chromatography)

- A measured quantity of urine whose pH is adjusted between 1.5 and 3.5 is neutralized and applied to a strongly basic anion exchange column.
- The column is washed to remove remaining interfering substances.
- The wash is followed by an elution buffer which eluates the VMA from the column.
- Two aliquots of the eluate are taken, one aliquot is used as sample for the VMA determination, the other as an individual sample blank.
- Carbonate buffer is added to all tubes to give a pH of approximately 11 and the VMA in the test tubes is oxidized to vanillin.
- The optical density of this complex is determined spectro-photometrically at 360 nm and 380 nm.
- The difference of the absorbance represents the content of VMA in the sample.

RESULTS

Results are tabulated in following (Tables 2,3 and 4). Table 2 shows the measures of central tendency and the dispersion for NSE, Ferritin, LDH, GGT and VMA

concentrations. Box and Whisker plots were plotted for NSE, ferritin, LDH, and VMA and the inter-quartile range was established for all the parameters studied. True

positive, true negative, false positive and false negative values at cut-off levels are shown in (Table 3).

Table 2: Measurement of central tendency and dispersion.

Parameters units	NSE (µg / l)		FER (ng/ ml)		LDH (U/L)		VMA (mg/g of creatinine)	
	I	II	I	II	I	II	I	II
Median	4	15.8	32.48	110.93	143.5	268	5	31.1
Minimum	1	3	5.93	15.91	76	74	1.2	5.24
Maximum	15	145	195.1	1857	230	5006	8	853.8
Interquartile range	1.5-5.5	5.3-79	16-104.6	46.2-196.82	104-190	147-160	3.5-6.95	17.0-118.1
p value	<0.00001*		0.001696*		0.000642*		0.00004	

*The result is significant at p <0.005 for NSE, ferritin LDH and VMA.

Table 3: Cut-off levels as well as true positives, true negatives, false positive and false negatives.

Parameters	NSE	FER	LDH	VMA
Cut off levels	4.4 (µg/l)	151 (ng/ml)	190 (U/L)	7 (mg/gm of creatinine)
True positive	79	26	55	89
True negatives	32	36	31	34
False positive	8	4	9	6
False negative	12	65	36	2

The sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of these cut-off levels are shown in (Table 4).

Table 4: Sensitivity, specificity, PPV and NPV.

Parameters	NSE	FER	LDH	VMA	MIBG
Sensitivity	90.00%	86.00%	85.90%	94.0%	70%
Specificity	72.00%	35.60%	46.20%	94.4%	100%
PPV	87.00%	28.50%	60.00%	97.8%	100%
NPV	80.00%	90.00%	77.50%	85.0%	66%

The sensitivity and specificity were calculated at different cutoffs levels. Cut off value selected was with maximum sensitivity and a stable level of specificity.

NSE levels in patients group showed mean value as 40.17±40.09 µg/L as compared to normal healthy controls with mean value 4.43±3.5 µg/L. it showed 90% sensitivity and 72 % specificity.

Mean value for Ferritin levels in Patients group was 216.32±327.77 ng/mL and normal healthy control group was 56.2±49.9 ng/mL with 86 % sensitivity and 35.6% specificity.

LDH levels showed 85.9% sensitivity and 46.2% specificity with mean value 560±789 U/L for patients' group and 148.17±48.2 U/L in normal control group.

VMA with highest sensitivity that is 94.0%, specificity 94.4% had mean value as 123.87±184.33 mg/gm of creatinine for patients' group and 5.08±1.93 mg/gm of creatinine for normal healthy control group.

Correlation

To measure the strength of correlation between VMA and all the studied parameters a Pearson correlation coefficient was used. The Pearson correlation is used to measure the strength of linear association between two variables, where the value r=1 means perfect correlation and r= -1 means a perfect negative correlation.

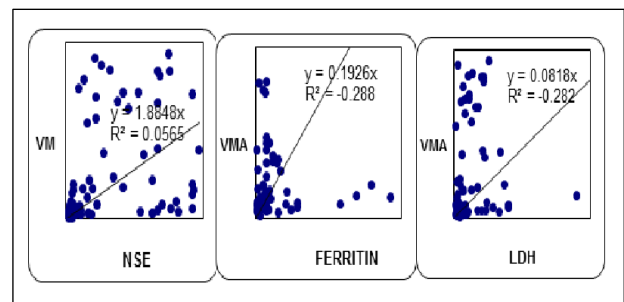


Figure 1: Following figures show the correlation between VMA to NSE, ferritin and LDH.

When VMA is compared with NSE, Ferritin, and LDH, it is noted that the r value is less than 0.5, although technically positive correlation, the relation between VMA and NSE or Ferritin or LDH is very weak.

DISCUSSION

Neuroblastoma is the fourth most common malignancy of childhood, preceded by leukemias, CNS tumors, and lymphomas. NB is the most common intra-abdominal malignancy of infancy and the most common extracranial solid tumor of childhood. It is a poorly differentiated

neoplasm derived from neural crest cells that typically affects infants and young children. NB is one of the small, blue, round cell tumors of childhood. Other such tumors include Ewing sarcoma, non-Hodgkin lymphoma, primitive neuroectodermal tumors, and undifferentiated soft tissue sarcoma (rhabdomyosarcoma).

“In children, the symptoms of cancer are often missed because they overlap with common illnesses. For example, we need to raise the red flag and suspect cancer if a child has fever for more than two weeks,” said Dr Sripad Banavali, head of TMH’s medical oncology unit.

“Cancer cases which relapse are candidates for bone marrow transplant. The cure rate of cancers with bone marrow transplant is 50%,” said Dr Naveen Khattri, who heads Tata Memorial Centre’s bone transplant unit.¹¹

Incidence of childhood cancer in India are as follows

Among 38-124 per ten lakh children per year. 40,000 new cancer cases seen in India each year.¹¹

Although cancer is the most common cause of disease-related death in children in the developed world, with improving survival rates, the mortality rate has declined to approximately 30 per million children per year. In India, the mortality rate (adjusted to world standard population) varies from 14 to 34 per million children per year, and on first glance appears similar or even better than the developed world. However, the incidence of childhood cancer in some areas of India, is much less than other parts of the world and the mortality: incidence (M: I) ratio rather than the mortality rate gives a more accurate picture of death from childhood cancer. This varies from 17 to 72% in India as compared to 20-24% for USA and Britain and is particularly high in rural Ahmadabad (61%) and Barshi (72%). Among the major urban areas, the mortality rate as well as M:I ratio in Mumbai is 1.5 to 2 times higher than that of Bangalore, Bhopal, Chennai, and Delhi. The reliability of these statistics depends on the comprehensiveness of death notification and quality of death certification, which are much higher in Mumbai than other areas of India. Therefore, data from Mumbai is probably a truer estimate of mortality in urban India.¹²⁻¹⁵

The aim of this work was to investigate the independent diagnostic value of different markers commonly evaluated in NB patients. Seventy nine of 91 patient’s specimens showed NSE activity levels greater than 4.4 µg/L, the most discriminate cut off level that was obtained by studying patients with NB and normal healthy controls. The levels of NSE were significantly elevated ($p < 0.001$) which is in confirmation with the findings of Schleiermacher Only twenty six of 91 specimens of patients showed Ferritin values higher than the cut off value 151 ng/mL and hence our results are not in agreement with Schleiermacher and Hann et al.^{16,17} Imashuku S and coworkers.¹⁰ They state that Serum

ferritin is elevated in most children with neuroblastoma who are in stages III or IV, but it is not elevated in those in stage I or II. In our study LDH was elevated in 57 out of 91 patients’ group with cut off levels 190 units/L. The results of study conducted by Pang QM and associates indicated that the positive rate of NSE in serum was high before treatment, and the levels of NSE and LDH were remarkably higher, which is in agreement with our study.¹⁸ Some other literature state that NSE and LDH level can be higher in Neuroblastoma cases. A study “Neuroblastoma workup” conducted by Byron D Joyner, MD, MPA; Chief Editor: Brian H Kopell, MD in July 2017 states that Neuron-Specific Enolase (NSE), Lactic Dehydrogenase (LDH), and ferritin are markers useful in the identification of active disease, as well as in prognostication.¹⁹

VMA had the best value at the optimal derived ROC cut off, that is 94.0% sensitivity, 94.4% specificity, 97.8% positive predictive value and 85.0% negative predictive value. VMA was elevated in 89 of 91 patients with NB and 2 of 40 healthy control group. Stephen J. Smith, in their study conducted on 14 Neuroblastoma cases state that urine catecholamine levels were found to be significantly elevated in 10 of 14(71%) cases of neuroblastoma.²⁰

Highlights of the study

At the optimal cut off level of 4.4 µg /l NSE showed 90% sensitivity, 72% specificity, 87% positive predictive value and 80% negative predictive value. Ferritin showed 86% sensitivity, 35.6 % specificity, 28.5 positive predictive value and 90% negative predictive value at cut off level of 151 ng/ml. 85.9% sensitivity, 46.2 % specificity, 60.0 positive predictive value and 77.5% negative predictive value were observed for LDH at optimal cut off levels of 190 units/L. At 46.0 units/L cut off level GGT showed 71% sensitivity, 30 % specificity, 10.9% positive predictive value and 90% negative predictive value. VMA showed highest sensitivity (91%), specificity (94.4 %) positive predictive value (97.8%) and 85% negative predictive value at the cut off levels of 7mg/ m of creatinine. MIBG scan shows 77% sensitivity, 100% specificity, 100% positive predictive value and 66% negative predictive value.

CONCLUSION

Results of this study suggests that the detection of VMA in combination with routine histological examination, MIBG scan, serum NSE and LDH may improve the diagnosis of NB. VMA as the conventional marker can be used as diagnostic indicator for NB.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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