STUDY OF SERUM BETA CATENIN LEVELS IN ACUTE MYELOID LEUKEMIA (AML) PATIENTS

Dr. Neeraj Yadav¹, Dr. Veena Singh Ghalaut² and Dr. Sudhir Kumar³

1. P.G. Student, Department of Biochemistry, Pt. B.D. Sharma PGIMS, Rohtak-12400, Haryana, India.
2. Senior Professor and Head, Department of Biochemistry, Pt. B.D. Sharma PGIMS, Rohtak-124001, Haryana, India.
3. Professor and Head, Clinical Hematology, Pt. B.D. Sharma PGIMS, Rohtak-12400, Haryana, India.

Manuscript Info

Introduction: Acute myeloid leukemia (AML) is a hematologic malignancy of the myeloid line of white blood cells in the bone marrow.

Aim and Objectives: To estimate serum levels of beta catenin in AML patients and in age and sex matched healthy controls.

Material and Methods: Twenty patients after confirmed diagnoses of acute myeloid leukemia were enrolled in the study. Diagnosis was based on blast cells 20% or more in peripheral blood smear/bone marrow by immunophenotyping (IPT)/flowcytometry. Twenty healthy age and sex matched volunteers served as controls.

Results: The mean age for patients was 36.95±15.28. The beta catenin (pg/mL) levels were increased in male AML patients before chemotherapy (115.41±26.22) as compared to male controls (19.66±5.43) (p <0.001). Beta catenin levels were also increased in female AML patients before chemotherapy (114.25±22.55) as compared to female controls (19.37±4.53) (p <0.001). Nine male patients (45%) and 7 female patients (35%) achieved remission after induction chemotherapy while 3 male patients (15%) and 1 female patient (5%) failed to achieve remission.

Conclusion: AML arise from a small pool of specialized leukemic stem cells (LSC), which have the idiosyncratic ability to self-renew. Targeted eradication of these stem cells will be crucial for permanent elimination of the disease and improved overall outcome. Wnt pathway controls self-renewal of hematopoietic stem cells (HSC) through regulation of beta catenin. Aberrant activation of beta catenin is a common event in AML and accumulating evidence indicates this pathway plays a critical role in the establishment and maintenance of myeloid neoplasms. Influencing this pathway seems to be a promising diagnostic and treatment strategy and should be followed up in further studies for future clinical use in patients with AML.
Introduction:-
Leukemias are malignant neoplasms of hematopoietic stem cells, characterized by diffuse replacement of the marrow by neoplastic cells. There is accumulation of abnormal white cells (neoplastic/leukemic) in the bone marrow leading to bone marrow failure, a raised circulating white cell count and infiltration of organs (e.g. liver, spleen, lymph nodes, brain). It is one of the leading cause of cancer deaths. Leukemia strikes more in adult than in children (10:1) and has slightly increased incidence in males than females (1-2:1).1

Leukemia can be acute or chronic depending upon cause of the disease. Acute leukemia is defined by the rapid increase of immature blood cells as a result of failure of bone marrow to produce normal cellular elements of blood resulting in anemia, hemorrhagic state due to thrombocytopenia and infections due to neutropenia.

Beta catenin is 90 kD multifunctional protein encoded by CTNNB1 gene and is involved in regulation and coordination of cell-cell adhesion and gene transcription. It was initially discovered in the early 1990’s as a component of a mammalian cell adhesion complex: a protein responsible for cytoplasmic anchoring.2 Beta catenin is a dual function protein involved in regulation and coordination of cell to cell adhesion and gene transcription. As beta catenin is a multi-functional protein its role is largely determined by its subcellular localisation. At the plasma membrane beta catenin is an important component of the adherens junctions and links cadherin adhesion proteins to the actin cytoskeleton. A second, cytoplasmic pool of beta catenin, acts as the primary effector of the canonical Wnt signalling pathway.3 The cellular level of beta catenin is mostly controlled by its ubiquitination and proteosomal degradation.

As a component of the cell adhesion complex, beta catenin can regulate cell growth and adhesion between cells. It may also be responsible for transmitting the contact inhibition signal that causes cells to stop dividing once the epithelial sheet is complete. Beta catenin is widely expressed in many tissues including cardiac, hepatic and colonic tissues. The highly conserved canonical Wnt/beta catenin signalling pathway plays a role in the proliferation, survival, differentiation and migration of cells during development, and plays a critical role in maintaining homeostasis in a range of adult tissues.4 Importantly, disruptions in this pathway have been implicated in the development of many diseases, most notably cancer.5

As beta catenin is a proto oncogene, mutation and over-expression of beta catenin is associated with many cancers including hepatocellular carcinoma, colorectal carcinoma, lung carcinoma, malignant breast tumors, ovarian and endometrial carcinoma.

Aberrant activation of beta catenin is a common event in AML and accumulating evidence indicates this pathway plays a critical role in the establishment and maintenance of myeloid neoplasms. In AML, increased beta catenin signalling has been associated with activating mutations in the fms like tyrosine kinase-3 (FLT3) receptor and the oncogenic AML1-eight-twenty one oncoprotein fusion (AML1-ETO) and promyelocytic leukaemia-retinoid acid receptor alpha (PML-RARα) translocation products. In the absence of these lesions, however, it remains unclear which mechanisms may activate beta catenin in AML more broadly. 6

As per available literature, there are very few studies on the role of serum beta catenin levels in acute myeloid leukemia patients in India to the best of our knowledge, thus, the present study was planned.

Materials and Methods:-
The present study was conducted in the Department of Biochemistry, in collaboration with the Department of Medicine (Clinical Hematology unit); Pt. B.D. Sharma PGIMS, Rohtak. Twenty patients after confirmed diagnosis of acute myeloid leukemia were enrolled in the study. Diagnosis was based on blast cells 20% or more in peripheral blood smear/bone marrow by immunophenotyping (IPT)/flowcytometry. Twenty healthy age and sex matched volunteers served as controls. Serum beta catenin levels were estimated along with complete haemogram in patients and age and sex matched healthy controls.

Sample collection and storage:
Venous blood was withdrawn from each subject using aseptic precautions in purple EDTA vacutainer for hematological investigation (analysed same day) and 4ml blood in red plain vacutainer from which serum was separated and stored at -20 0 C for analysis of serum beta catenin.
Estimation of beta catenin:
Serum beta catenin was assayed by Enzyme Linked Immunosorbent Assay (ELISA).7

Principle:
The ELISA kit uses sandwich-ELISA as the method. The microelisa stripplate provided in this kit has been pre-coated with an antibody specific to beta catenin. Standards or samples are added to the appropriate microelisa stripplate wells and combined to the specific antibody. Then a horseradish peroxidase (HRP) conjugated antibody specific for beta catenin is added to each microelisa stripplate well and incubated. Free components are washed away. The 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution is added to each well. Only those wells that contain beta catenin and HRP conjugated beta catenin antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of beta catenin. Concentration of beta catenin in samples can be calculated by comparing the OD of the samples to the standard curve.

Statistical analysis:
IBM SPSS ver. 20 was used for various statistical analysis. Student t-test was applied to the data confirming to normal distribution. Correlation coefficient (r) was used to determine the relationships between different quantitative values. Statistical analysis was expressed by mean + standard deviation. For all tests a probability <0.05 was considered significant. Charts and graphs were prepared using IBM SPSS ver. 20 and Microsoft Excel programs.

Observations:-
Age distribution was similar in male AML patients and controls (p=0.952) and female AML patients and controls (p=0.988). The mean age for patients was 36.95±15.28. The Hb (g/dL) levels were significantly decreased in both male (6.84±1.42) and female AML patients (5.5±1.66) as compared to male controls (14.11±1.43) and female controls (13.59±1.15) and found to be statistically highly significant (p <0.001). The TLC (cells/µL) levels were increased in male AML patients (25000±11862.85) as compared to male controls (7083.33±1505.04) and found to be statistically highly significant (p<0.001). TLC levels were also increased in female AML patients (22687.50±9307.97) as compared to female controls (7550±2061.20) and found to be statistically highly significant (p<0.001).

Table 1: Comparison of beta catenin in AML patients and control group.

|                | Males |        | Females |        |
|----------------|-------|--------|---------|--------|
| Beta catenin   |       |        |         |        |
| (pg/mL)        |       |        |         |        |
| AML (n=12)     | 115.41±26.22 | 19.66±5.43 | <0.001 | 114.25±22.55 | 19.37±4.53 | <0.001 |
| Control (n=12) | 107.5 | 19     | 111.50  | 19     |
|                 | Range | 73-163 | 12-28   | 86-150 | 14-25   |

The beta catenin (pg/mL) levels were increased in male AML patients (115.41±26.22) as compared to male controls (19.66±5.43) and found to be statistically highly significant (p <0.001). Beta catenin levels were also increased in female AML patients (114.25±22.55) as compared to female controls (19.37±4.53) and found to be statistically highly significant (p <0.001).

Table 2: Mean comparison of various parameters in AML patients and control group.

|                  | Group I (AML)) | Group II (Control) | Gr. I vs. II |
|------------------|----------------|--------------------|--------------|
| Hb (g/dL)        | 7.50±1.70      | 13.8±1.26          | <0.01        |
| TLC (cells/µL)   | 24075 ± 10711.86 | 7270 ± 1712.21     | <0.01        |
| Platelet count   | 52500 ± 24095.20 | 248750 ± 64110.82  | <0.01        |

Mean Hb (g/dL) comparison showed that group II had higher mean Hb level as compared to group I i.e. 13.8±1.26 and 7.50±1.70 respectively. On statistical analysis the difference among Gr. I vs. II found to be significant (p <0.01) and Gr. I vs. II found to be insignificant (p=0.120). Mean TLC (cells/µL) comparison illustrates that group I had higher mean TLC level as compared to group II i.e. 24075±10711.86 and 7270±1712.21, respectively. On statistical
analysis the difference among Gr. I vs. II found to be significant (p <0.01). Mean platelet count (cells/ µL) comparison demonstrates that group II had higher mean platelet count as compared to group I i.e. 248750±64110.82 and 52500±24095.20, respectively. On statistical analysis the difference among Gr. I vs. II found to be significant (p <0.01).

**Table 3:** Mean beta catenin levels in two groups.

| Beta catenin (pg/mL) | Group I (AML) | Group II (Control) | Gr. I vs. II |
|----------------------|--------------|--------------------|--------------|
| Mean±SD              | 114.95 ± 24.20 | 19.55 ± 4.96       | <0.01        |

Mean beta catenin (pg/mL) comparison showed that group I had higher mean beta catenin as compared to group II i.e. 114.95±24.20 and 19.55±4.96, respectively (p<0.01).

Nine male patients (45%) and seven female patients (35%) achieved remission after induction chemotherapy while three male patients (15%) and one female patient (5%) failed to achieve remission.

**Table 4:** Comparison of before chemotherapy beta catenin levels with treatment outcome.

| Beta catenin (pg/mL) | Remission (n=16) | Treatment failure (n=4) | p value |
|----------------------|------------------|-------------------------|---------|
| Mean±SD              | 106.25±17.14     | 149.75±14.93            | <0.01   |
| Median               | 106.5            | 151                     |         |
| Range                | 73-150           | 134-163                 |         |

Before chemotherapy beta catenin (pg/mL) levels were lower in patients who achieved remission (106.25±17.14) than in patients in which treatment failed (149.75±14.93) and statistically the difference among both groups found to be significant (p <0.01).

**Discussion:**

In the present study out of 20 AML cases; 12 were males (60%) and 8 were females (40%). The distribution of females and males were similar in cases and controls. In our study at the time of diagnosis the male patients (n=12) had a median age 31 years and female (n=8) patients had a median age 36 years. Similar age distribution was observed by Philip et al in AML (excluding acute promyelocytic leukemia) patients where the median age of newly diagnosed patients was 40 years and there were 64 % males.8 The median age of 31 years (range: 14–61) in our study is strikingly different from that routinely reported in the literature from developed countries. This lower median age could be due to a combination tertiary centre referral bias and a different population pyramid structure in India and other developing countries, where the proportion of people over the age of 60 years is significantly lower. However, the possible role of additional genetic and environmental factors cannot be excluded.

The Hb levels were significantly decreased in both male and female AML patients as compared to controls (p <0.001). Anemia is a constant feature in all acute leukemias. Anemia, in the present study correlates well with the studies done by Preethi and Mathur et al.9,10 Anemia in majority of cases is due to bone marrow infiltration leading to decreased production of red blood cells. Rarely anemia may occur due to decreased red cell life span and autoimmune destruction. Disturbed hematopoiesis leads to the most common presenting manifestations of AML such as anemia, infection and bleeding tendency.

The TLC levels were significantly increased in AML patients as compared to controls (p<0.001). In the present study, TLC ranged between 10000 – 45000 cells/µL with a mean of 24075±10711.8 cells/µL. In studies conducted by Mathur et al the TLC range was between 5000-100000 cells/µL with a mean of 385000 cells/µL.10 Mean platelet count in AML patients was lower as compared to controls and this difference found to be statistically highly significant (p <0.001). Similar results were reported by Preethi and Mathur et al in AML patients.9,10 Gaydos et al were the first to document thrombocytopenia in patients with AML and they also established a linear relationship between bleeding and platelet count. 11

Mean beta catenin (pg/mL) levels are higher in group I as compared to group II (p <0.01). Before chemotherapy beta catenin levels were lower in patients who achieved remission than in patients in which treatment failed and
statistically the difference among both groups found to be significant (p <0.01). Hb levels were negatively correlated with beta catenin levels in AML patients (r= -0.307) but statistically found to be insignificant (p=0.189).

The Wnt/ beta catenin pathway has been shown to play a critical role in the regulation of cell proliferation, differentiation and apoptosis of different malignant entities. Wnt/beta catenin signaling has a critical role in the regulation of self-renewal, proliferation and differentiation of cells in stem-cell niches, including the skin, hair follicle, the mammary gland, the intestinal crypt and the bone marrow. The aberrant activation of this signaling has been widely associated with tumorigenesis in these tissues. Mutations in beta catenin and other components of the Wnt signaling pathway including axin and TCF4 have been detected in a range of other cancers including hepatocellular carcinoma, melanoma, and gastric and ovarian cancers.12

Wnt pathway controls self-renewal of hematopoietic stem cells (HSC) through regulation of beta catenin. In HSC, beta catenin also modulates survival and growth factor response both in vitro and in vivo, while inhibiting differentiation. The importance of beta catenin in the maintenance of the HSC pool echoes in many hematological malignancies of lymphoid or myeloid origin. In leukemia evidence suggested that aberrant activation of Wnt/beta catenin signaling pathway may be a common event in aberrant hematopoiesis. In contrast to solid cancers, mutations in beta catenin or its central regulatory proteins are rarely identified in leukemias indicating that additional non-canonical mechanisms underlie beta catenin stabilization in leukemic individuals. Studies demonstrated that exposure of primitive hematopoietic cells to Wnt ligands significantly increases their proliferation in vitro and maintained the immature phenotype of these cells. Accordingly, Wnt ligands and receptors were shown to be expressed in the HSC and these proteins are also expressed in the cells that make up the bone marrow microenvironment. Extrinsic signals emanating from the microenvironment niche are crucial in the regulation of HSC self-renewal and quiescence.13

The Wnt/beta catenin pathway was found to be one of the key signalling networks deregulated in leukemic stem cells (LSC) compared to normal haematopoietic stem cells (HSC). Increased beta catenin nuclear localisation in AML LSC compared to normal progenitors as seen in flow-cytometry imaging indicates that this pathway may play an active role in the LSC compartment. Aberrant beta catenin expression in AML might suggest the presence of a subpopulation of AML cells that harbor “leukemic stem cells (LSC)” activity which will be difficult to eradicate by modern chemotherapeutic agents and hence lead to poor survival.

Muller et al first identified a role of Wnt/beta catenin pathway in the pathogenesis of AML.14 Activating mutations of FLT3 was found to synergize with Wnt-dependent signaling in myeloid transformation and leukemogenic effects. In normal hematopoiesis, beta catenin is mainly expressed in primary CD34 progenitor cells but is rapidly lost upon differentiation.

Ysebaert et al performed methylcellulose based colony formation and replating assays with primary AML samples which were classified for the presence or absence of beta catenin. Ysebaert et al stated that Wnt signaling is deregulated in AML as assessed by beta catenin expression which is rapidly lost upon induction of differentiation in normal precursors and is correlated to enhanced clonogenic proliferation of leukemic colony forming cells (CFU-L). They also reported that beta catenin expression appears as a new prognostic factor independently correlated with relapse and shorter survival in AML patients. They also suggested that high clonogenic capacities and increased self-renewing potentials of LSCs driven by aberrant beta catenin expression can lead to enhanced aggressiveness and/or rapid recurrence of the disease.15

Jamieson et al have shown that aberrant activation of the Wnt/beta catenin pathway results in enhanced self-renewal capability in granulocyte-macrophage progenitors of blast-crisis CML.16 These cells express significantly higher levels of beta catenin mRNA and protein than normal hematopoietic progenitors. Constitutive activation of Wnt/ beta catenin pathway has been reported in a significant proportion of AML cases and correlates with increased blast clonogenicity and poor outcome.16

Juan-Xaio et al in their study on serum beta catenin levels associated with the Ratio of RANKL/OPG in Patients with Postmenopausal Osteoporosis reported that serum beta catenin levels in healthy postmenopausal controls were 39.33±5.47 pg/mL which is comparable to our study.17
Recent studies reported that reduction of intracellular beta catenin levels in AML cell lines and patient samples decreased their rate of proliferation in vitro without affecting cell viability in contrast to normal human CD34+ progenitor cells. This downregulation of beta catenin causes a G1/G2 phase increase of cells while lowering the amount of S phase cells. Overexpression of beta catenin seems to be an independent adverse prognostic factor in AML. Overexpression of beta catenin is associated with both shortened relapse-free survival and poor overall survival suggesting that AML associated with aberrant beta catenin signaling may represent a particularly aggressive disease.15

Recently Kim et al reported that when the intracellular concentration of beta catenin was downregulated by a short hairpin RNA lentivirus the proliferation of leukemic cell lines deteriorates. In contrast some authors, suggests that despite effective downregulation of beta catenin in primary AML cell lines, a decreased proliferation rate could not always be observed. Thus, targeted therapy against beta catenin might not be successful in all patients.19

Abnormal promoter methylation of specific Wnt/beta catenin inhibitors has also been reported in myeloid malignancies and correlates with poor prognosis in patients highlighting that beta catenin activation is a common downstream target of a variety of AML associated lesions. It suggests that beta catenin can be an attractive therapeutic target in this disease. The activation of prostaglandin E2 (PGE2) by its upstream regulator cyclooxygenase (COX) has been previously shown to induce the stabilization of beta catenin in colon cancer, HSC and leukemic cells suggesting this may be an active mechanism for beta catenin regulation in disease development.19

The studies conducted so far on beta catenin expression in AML patients conclude that Wnt / beta catenin pathway plays a major role in pathogenesis of acute leukemia. In spite of divergent results in various studies, influencing this pathway seems to be a promising treatment strategy and should be followed by in further studies for future clinical use in patients with AML.

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