Original Research Article
Relationship Between E3 SUMO-Protein Ligase NSE2 (NSMCE2) with Ecto-5'-nucleotidase, ADA and AMPDA Enzymes in Patients with Atherosclerosis

ABSTRACT

Objective: the aim of this study were to determine the level of the E3 SUMO-Protein Ligase (NSMCE2) in pg/ml and their relationship to Ecto-5'-nucleotidase, ADA and AMPDA enzymes U/l in Patients with atherosclerosis compared with healthy normal.

Method: The level(pg/ml) of NSMCE2 was assay quantitatively by using sandwich enzyme immunoassay technique. The activity in U/L for Ecto-5'-nucleotidase, AMPDA and AMPDA were measured in serum of control and patients with atherosclerosis.

Results: The present study included 60 male patients with atherosclerosis and thirty males matched apparently healthy individuals as control group. The present study showed that mean levels of sera NSMCE2(219.25±52.04 pg/ml) have a highly significantly increase (p<0.0001) in patients compared to control group (101.82±23.20 pg/ml), while the serum mean value activates in U/L for Ecto-5'-nucleotidase, ADA and AMPDA in patients were significantly increased with values of 49.05±18.08, 41.74±16.89 and 39.06±15.03 U/l, respectively compared with the activity of healthy control having the vales of 12.15 ±3.97, 14.35 ±3.01 and 12.53 ±4.20, respectively.

Conclusion: measuring of E3 SUMO-Protein Ligase (NSMCE2) in pg/ml and the activates of Ecto-5'-nucleotidase, ADA and AMPDA enzymes U/l in patients with atherosclerosis highly significant elevation, compared with healthy normal. This normally expressed in vascular endothelial cells and a broad range of immune cells.
Therefore, adenosine formed by extracellular nucleotide catabolism on endothelial cells and immune cells appears to be an important endogenous modulator of arteriogenesis and key transcription factors involved in inflammatory responses.

**KEYWORDS:** Atherosclerosis, E3 Sumo-Protein Ligase, NSMCE2, Ecto-5'-nucleotidase, ADA, AMPDA

**INTRODUCTION**

Atherosclerosis is a slowly progressing and multifactorial disease, in which endothelial dysfunction and damage play an initial role. Many risk factors for atherosclerosis can lead to endothelial damage of the vessel, especially in the areas where blood flow is disturbed. In the presence of hyperlipidemia, disturbed blood flow results in increased endothelial turnover in the arterial wall. It was demonstrated that disturbed blood flow activates endoplasmic reticulum stress initiating a signal pathway leading to endothelial apoptosis. Following endothelial death, the neighboring mature endothelial cells actively proliferate and migrate to heal the wound.  

Small Ubiquitin-like Modifier (SUMO) proteins are a family of small proteins belongs to the ubiquitin (Ub) and ubiquitin-like (Ubl) protein family. The SUMO proteins are small; most are around 100 amino acids in length and 12 kDa in mass. The exact length and mass varies between SUMO family members and depends on which organism the protein comes from. Sumo shares only 18% sequence homology with ubiquitin. Although SUMO has very little sequence identity with ubiquitin at the amino acid level, it has a nearly identical structural fold (structurally similar to ubiquitin). SUMO proteins are covalently attaches to certain lysine residues of specific target proteins in cells and alters a number of different functions depending on the substrates. The SUMOylation is a dynamic and reversible process regulated by both conjugation and de-conjugation enzymes via a three-step process and three
enzyme reactions, E1 (SUMO-activation enzyme), E2 (SUMO conjugation enzyme), and E3 (SUMO ligase). SUMOylation is a part of important regulatory mechanisms that modify proteins in the nucleus and regulate multiple cellular processes such as nucleo-cytoplasmic signal transduction, apoptosis, stress responses, protein stability, subcellular localization of proteins, protein-protein interactions, protein-DNA interactions, and transcriptional activity of transcription factors and progression through the cell cycle\textsuperscript{5,6}.

Ecto-5'-nucleotidase is an enzyme that hydrolyzes extracellular AMP to adenosine and represents the major control point for extracellular adenosine levels and is a regulator of the adenosine signaling pathway. Ecto-5'-nucleotidase is an intrinsic membrane glycoprotein, present as an ectoenzyme in a wide variety of mammalian cells and it belongs to a conserved superfamily of metallophosphodiesterases. Extracellular and intracellular Ecto-5'-nucleotidase activities regulate the quantity of nucleotides generated from both de novo and salvage pathways and participate in purine salvage to support balanced synthesis of nucleotides, which is critical for maintaining high fidelity DNA replication\textsuperscript{7,8}.

Adenosine aminohydrolase (ADA) is a polymorphic enzyme involved in purine metabolism and it is essential in the purine salvage pathway\textsuperscript{9}. The ADA catalyzes the irreversible hydrolytic cleavage (deamination) of adenosine to inosine and ammonia. The enzyme is widely distributed in animal and human tissues. It is present in the cytoplasmic fraction and a certain amount is located in the nucleus\textsuperscript{10,11}. Although found in most tissue, ADA activity is greatest in lymphoid tissue. Its activity is 10-20 times more active in T lymphocytes than in B lymphocytes and it is necessary for the proliferation, maturation and function of lymphocytes, specifically for T lymphocytes and the maturation of monocytes to macrophages\textsuperscript{11,12}.
The AMP-aminohydrolase (AMPDA) is a key enzyme of nucleotide breakdown involved in regulation of adenine nucleotide pool in the liver and energetic metabolism in mammalian cells. Also, it plays a crucial role in the synthesis of guanine nucleotides and in the provision of anaplerotic substrates for the Krebs cycle. AMPDA is an enzyme that converts AMP to IMP, freeing an ammonia molecule in the process. It has several unique cellular functions and its activity and expression pattern are highly tissue specific. It plays an important role in the purine nucleotide cycle, which is designed to preserve adenylate's energy charge and phosphorylation potential under conditions of insufficient energy supply.\textsuperscript{13}

**MATERIALS AND METHODS**

This study was conducted on a cohort of 60 patients with atherosclerosis and 30 healthy persons to be used as control ranging between (40-75) years. These patients were hospitalized at Research Institute for educational laboratories in the city of Medicine of the Ministry of Health. Five milliliter of blood sample were collected and centrifuged at [3000 rpm] for 5 min. The resultant serum were separated and stored at [-20°C] until used. The NSMCE2 assay employs the quantitative sandwich enzyme immunoassay technique by CUSOBIO kit. Ecto-5'-nucleotidase activity was measured in serum according to Wood and Williams's method.\textsuperscript{14} Adenosine Aminohydrolase (ADA) activity was determined by Giusti and Galanti method.\textsuperscript{15} Determination of AMPDA activity was carried out using the same procedure that was used for ADA activity except changing the buffer and substrate used, since AMPDA activity has an absolute requirement for $K^+$ ions.

**RESULTS AND DISCUSSIONS**

The present study included sixty male patients with atherosclerosis and thirty males matched apparently healthy individuals as control group. The present study showed
that mean levels of sera NSMCE2 have a highly significantly increase ($p<0.0001$) in patients group compared to control group as shown in Table 1 and Figure 1.

Table 1: The mean and standard deviation of serum NSMCE2 [pg/ml] in patients and control groups.

| Studied group       | No. | Mean ± SD  | Range          | Comparison of Significant |
|---------------------|-----|------------|----------------|---------------------------|
| Patients group      | 60  | 219.25±52.04 | 98.52-337.98 | Highly Significant [p<0.0001] |
| Control group       | 30  | 101.82±23.20 | 62.02-155.22 | 0.0001                    |
| Total               | 90  |             |                |                           |

Figure 1: Values for NSMCE2 [pg/ml] in patients and control group

The small ubiquitin-related modifier (SUMO) system has been implicated in numerous physiological and pathological processes through altering the functions of its target proteins. The SUMO covalent linkage is usually through the lysine residue(s). Some sumoylation assays revealed that in the presence of E1 and E2, the E3 ligase was dispensable to accomplish SUMO conjugation. However, SUMO E3 ligases contributed to the efficiency and specificity of SUMO conjugation and were attributed to the RING domain, which is similar to the corresponding structure in E3 ligases involved in the ubiquitination. Several reports refer that SUMO modification activated several cardiac muscle-restricted genes.

Recent studies indicate a role for sumoylation in the regulation of inflammation that is initiated in response to tissue damage and infectious agents. Inflammatory
responses must be regulated properly, and unrestricted inflammation can lead to inflammatory disorders.\textsuperscript{18}

Atherosclerosis is considered to be a chronic inflammatory disease.\textsuperscript{19} The transcriptional induction of genes involved in inflammatory responses is controlled by various transcription factors, including nuclear factor κB (NF-κB), signal transducer and activator of transcription (STAT) and activator protein-1 (AP-1). Sumoylation can regulate inflammation through the direct modulation of the activity of key transcription factors involved in inflammatory responses.\textsuperscript{20,21}

A member of the protein inhibitor of activated STAT (PIAS) family, PIAS1, which possesses SUMO E3 ligase activity,\textsuperscript{22} is a transcriptional repressor of NF-κB and STAT1. PIAS1 functions by blocking the DNA-binding activity of NF-κB and STAT1 on gene promoters. Recent studies indicate that PIAS1 is activated by phosphorylation in response to pro-inflammatory stimuli, a process that requires the SUMO ligase activity of PIAS1. Activated PIAS1 is then recruited to inflammatory gene promoters to repress NF-κB and STAT1-mediated transcription. These findings support a hypothesis that targeting the PIAS1 sumoylation pathway might represent a novel therapeutic strategy for the treatment of inflammatory disorders such as atherosclerosis.\textsuperscript{23}

The activity and specific activity of serum ecto-5'-nucleotidase showed a highly significant increase ($p<0.001$) in patients group compared to control group as shown in Table 2 and Figure 2.
Table 2: The mean and standard deviation of serum Ecto-5'-nucleotidase [U/L] in patients and control groups.

| Characteristics         | Patients group [n=60] | Control group [n=30] | Comparison of Significant |
|-------------------------|-----------------------|----------------------|--------------------------|
|                         | Mean ± SD             | Mean ± SD            | p Value                  | Sig                  |
| Activities [U/L]        | 49.05±18.08           | 12.15 ±3.97          | 0.0007                   | Highly-Significant [p<0.001] |
|                         | 25.19-119.84          | 5.34-21.37           |                          |                      |
| Specific Activities [U/mg] | 0.72±0.29             | 0.17±0.05            | 0.0004                   | Highly-Significant [p<0.001] |
|                         | 0.35-2.02             | 0.08-0.30            |                          |                      |

Table 3 and Figure 2 illustrate the activity and specific activity of serum ADA showed a highly significant increase (p<0.001) in patients group in comparison to control group. Activity and specific activity of serum AMPDA in patients group are compared to control group as shown in Table 4 and Figure 2. In general, the activity of AMPDA in patient group showed highly significant (p<0.001) increase compared to control group.
Ecto-5'-nucleotidase is important membrane-bound enzyme involved in the metabolism of extracellular nucleotides\textsuperscript{24,25}. It catalyzes the hydrolysis of AMP generating adenosine that is a potent vasodilator and anti-inflammatory molecule and participates in numerous important biological functions and physiological effects, such as mediation of tubuloglomerular feedback, playing a crucial role in hypoxia-induced vascular leakage, acts as an endogenous modulator protecting against vascular inflammation and monocyte recruitment to limit the progression of atherosclerosis, and enable the efficient entry of lymphocytes into the central nervous system during autoimmune encephalitis\textsuperscript{25,26}. It is becoming increasingly apparent that

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\textbf{Characteristics} & \textbf{Patients group [n=60]} & \textbf{Control group [n=30]} & \textbf{Comparison of Significant} \\
\hline
\textbf{Activities [U/L]} & & & \\
Mean ± SD & 41.74±16.89 & 14.35 ±3.01 & 0.0008 \text{Highly-Significant} \text{[p<0.001]} \\
Range & 16.44-78.00 & 10.00-21.00 & \\
\hline
\textbf{Specific Activities [U/mg]} & & & \\
Mean ± SD & 0.61±0.26 & 0.20±0.04 & 0.0009 \text{Highly-Significant} \text{[p<0.001]} \\
Range & 0.22-1.22 & 0.13-0.28 & \\
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\caption{The mean and standard deviation of serum ADA [U/L] in patients and control groups}
\end{table}

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\textbf{Characteristics} & \textbf{Patients group [n=60]} & \textbf{Control group [n=30]} & \textbf{Comparison of Significant} \\
\hline
\textbf{Activities [U/L]} & & & \\
Mean ± SD & 39.06±15.03 & 12.53 ±4.20 & 0.0005 \text{Highly-Significant} \text{[p<0.001]} \\
Range & 23.00-77.56 & 4.03-19.23 & \\
\hline
\textbf{Specific Activities [U/mg]} & & & \\
Mean ± SD & 0.57±0.23 & 0.17±0.06 & 0.0006 \text{Highly-Significant} \text{[p<0.001]} \\
Range & 0.30-1.13 & 0.06-0.27 & \\
\hline
\end{tabular}
\caption{The mean and standard deviation of serum AMPDA [U/L] in patients and control groups}
\end{table}
adenosine plays a central role in the regulation of the inflammatory response. Ecto-5'-nucleotidase is an enzyme normally expressed on vascular endothelial cells and a broad range of immune cells. Therefore, adenosine formed by extracellular nucleotide catabolism on endothelial cells and immune cells appears to be an important endogenous modulator of arteriogenesis, so it is obvious that the role of ecto-5'-nucleotidase-derived adenosine in a model of chronic vascular inflammation such as atherogenesis. This establishes Ecto-5'-nucleotidase-derived adenosine as a direct or indirect regulator of atherogenesis.

In present study, a highly significant increase in ecto-5'-nucleotidase levels in atherosclerosis patients explains that its adenosine can convey protection against atherosclerosis, an interference with intrinsic physiological pathways involved in the adenosine metabolism and ecto-5'-nucleotidase is crucially involved in the finely tuned constitutive regulation balancing proinflammatory in the microvasculature.

Adenosine aminohydrolase also has effect on the activation of complement system by the deamination of adenosine. The ADA catalyzes the conversion of adenosine to inosine, so adenosine aminohydrolase associates with and alter local concentrations of adenosine. The major source of serum ADA may be lymphocytes or the monocyte-macrophage cell system. It was reported that elevated levels of ADA reflect the changes in the immune response in the pathogenesis of atherosclerosis and coronary heart disease. Hence ADA can be considered as important marker in assessing atherosclerosis and coronary heart disease. Also, ADA is considered as an inflammatory marker. Thus, ADA has a role in coronary artery disease by affecting inflammation process. Second, many effects produced by ADA are caused by the metabolism of adenosine. It is known that adenosine can inhibit the invasion of the neutrophil so as to attenuate the ischemic/reperfusion injury. Adenosine can increase
coronary artery blood flow during active stress and hypoxia to balance the oxygen supply and demand. Adenosine can also account for the majority of basal vascular endothelial growth factor and protein expression in cultured myocardial vascular smooth muscle cells under normoxic conditions to stimulate the angiogenesis. If adenosine is rapidly metabolized by the high level of ADA, the advantages of adenosine will lost. Finally, adenosine is catalyzed to inosine, which can produce superoxide radicals and exaggerate the ischemic/reperfusion injury. AMP-aminohydrolase is the rate-limiting step for entry into the purine nucleotide cycle and catalyzes the conversion of adenosine monophosphate (AMP) to inosine monophosphate (IMP). A genetic background to the diversity seen in the clinical progression of heart disease is well documented. Genetic diversity in pathways involving nucleotide metabolism are particularly important due to the latter’s direct links to myocardial function and metabolic regulation. Several polymorphisms of the AMPDA gene may be associated with progression of the heart disease.

Previous studies Safranow did not find significant differences in the AMPDA polymorphism genotype distributions in the study groups of heart failure and coronary artery disease and in a random control group. It could be interpreted as a protective effect of the T allele against cardiovascular diseases, but such a hypothesis should be treated with caution due to the low moderate statistical significance. The precise mechanism of the beneficial clinical effect of the AMPDA mutation is uncertain. In spite of the increased production of adenosine, a product of the alternative pathway of AMP catabolism has been suggested. Adenosine exerts numerous effects which can attenuate the progression of heart and ischemic heart disease, such as vasodilatation, inhibition of platelet adherence, anti-adrenergic regulation of the inflammatory and the immune system. The elevation in the activity of AMPDA
might be associated with intima-media thickness of the carotid and brachial artery, endothelial function of the brachial artery, glucose metabolism, haemostatic variables and cardiac hypertrophy in patients with coronary heart disease, which need further study.

In conclusion, according to this study, the increase in NSMCE2 may play a role in developments of change DNA damage in the patients with atherosclerosis. Increased in ecto-5'-nucleotidase activity also recorded in this study, that may resulted from the effect of elevation in lipid peroxidation on the permeability of cell membrane since ecto-5'-nucleotidase is a membrane bound protein. The present study suggests that ADA may serve as an indicator of underlying inflammation, and ADA can have an important role in coronary artery disease, if design and development of therapeutic strategies against ADA are guaranteed; an innovational therapeutic method to atherosclerosis disease can be recognized. The elevated AMPDA leading to reduce the adenosine level, by converted AMP to IMP in the serum of the patients, which might confirm the atherosclerosis status, which need further study the average value increments of IMP in atherosclerosis patients. As our knowledge, no previous studies have showed these results in atherosclerosis patients.

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