Role of calpain and caspase system in the regulation of N-myristoyltransferase in human colon cancer (Review)

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Received October 18, 2006; Accepted December 1, 2006

Abstract. A number of viral and eukaryotic proteins which undergo a lipophilic modification by the enzyme N-myristoyltransferase (NMT: NMT1 and NMT2) are required for signal transduction and regulatory functions. We reported a higher expression of NMT2 in most of the cases of cancerous tissues compared to normal tissues by Western blot analysis. Furthermore, protein-protein interaction of NMTs revealed that *m*-calpain interacts with NMT1 while caspase-3 interacts with NMT2. Our findings provide the first evidence of higher expression of NMT2 in human colorectal adenocarcinomas and the interaction of both forms of NMT with various signaling molecules. In this review, we summarize the recent findings on NMT2 in human colon cancer in our laboratory.

Contents

1. Introduction

Myristoylation is a co-translational lipid modification of proteins (1) in which myristate (a 14-carbon fatty acid) is covalently attached to the amino terminus of various cellular, viral and onco proteins (2-8). Cellular myristoylated proteins have diverse biological functions in signal transduction and oncogenesis (2-8). The processes of myristoylation is catalyzed by the ubiquitously distributed eukaryotic enzyme N-myristoyltransferase (NMT), which is a member of Glycylpeptide N-tetradecanoyltransferase (GNAT, EC 2.3.1.97) (2-8). NMT exists in several distinct forms varying in either apparent molecular weight and/or subcellular distribution (9-13). King and Sharma (9) provided the first evidence for the existence of multiple forms of bovine brain NMT. Later, McIlhinney *et al* (10) also identified two forms of NMT in bovine brain cortex. Subsequently, Glover and Felsted (11) showed that bovine brain NMT exists as a heterogeneous mixture of NMT subunits.

The gene for the NMT enzyme is ubiquitous in all eukaryotes, sharing highly conserved domains across species as diverse as *Saccharomyces cerevisiae* and humans (14-16). Mammals and plants have two genes for NMT, NMT1 and NMT2, while other species such as the fruit fly appear to have only one (12,17-19). The predicted 496-amino acid human NMT1 protein shares 77 and 97% sequence identity with human NMT2 and mouse NMT1 respectively, indicating that NMT1 and NMT2 represent two distinct families of NMT (12). Two types of NMT, NMT1 and NMT2, were identified and cloned from various sources (12,13).

2. NMT in cancer

Protein myristoylation is a co-translational process that occurs after the removal of methionine. However, there have been reports documenting post-translational myristoylation of protein such as the pro-apoptotic protein BID (20,21). Methionine aminopeptidase (MetAP) is the enzyme responsible for the removal of methionine from the amino-terminus of newly synthesized proteins (22). We observed elevated MetAP2 expression for the first time in human colorectal adenocarcinomas and various human colon cell lines (HCCLs) (23,24). In addition, we have demonstrated the differential expressions of $pp60^{c\text{-}src}$, MetAP2 and NMT1 in HT29, human colon cell line (24).

C-Src is overexpressed in a number of human cancers, especially those of colon (25,26) and breast (27). The tyrosine kinase activities of N-myristoylated $pp60^{c\text{-}src}$ and $pp62^{c\text{-}yes}$ protein kinases are significantly elevated in primary colorectal adenocarcinoma as well as in their corresponding cell lines relative to those of normal cells (26-28). Earlier, we reported that NMT activity is higher in colonic epithelial neoplasms than in normal appearing colonic tissue and that increased

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Key words: lipid modification, N-myristoyltransferase 2, *m*-calpain, caspase-3, p53, Bcl2

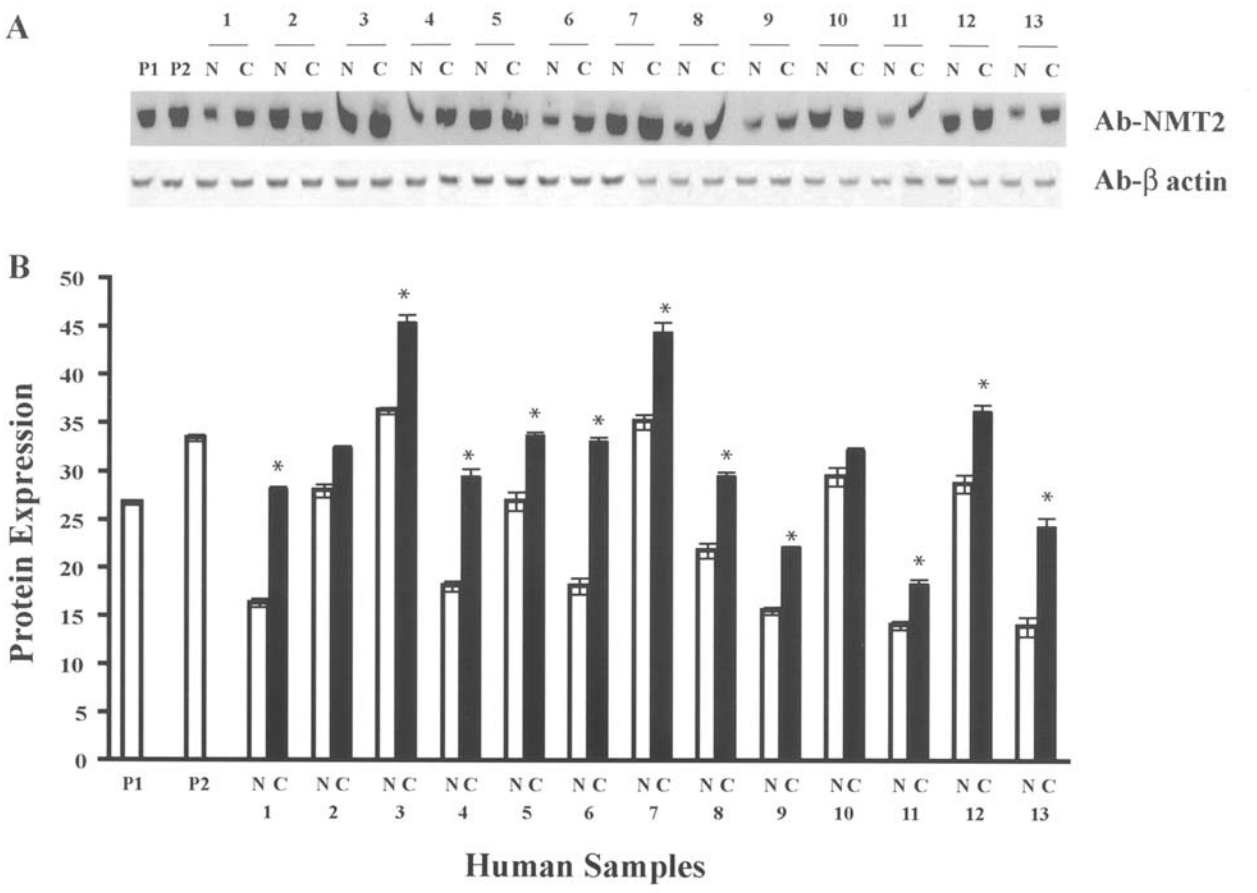

Figure 1. (A) Western blot analysis of N-myristoyltransferase 2 in human colorectal carcinoma. Polyps (P), normal (N), and tumor tissue (C); (B) Quantitation analysis of A was carried out using image software (NIH at http://rsb.info.nih.gov/nih-image/download.html). For details see (35).

NMT activity appears at an early stage in colonic carcinogenesis (29). Furthermore, we observed that the NMT expression is elevated in human colon (30) and gallbladder carcinomas (31). Recently, we reported high expressions of both NMT1 and NMT2 in human brain tumors (32). We discovered and characterized an NMT inhibitor protein 71 (NIP71) (33). Furthermore, we identified that the NIP71 is homologous to heat shock cognate protein 70 (Hsc70) (34). Our previous studies have focused on NMT1 and its expression in various tumors. To our knowledge, no studies are available related to NMT2 in colorectal adenocarcinomas.

3. Expression of NMT2 in human colorectal tissues

We investigated the expression of NMT2 in colorectal carcinoma and observed a high expression of NMT2 in human colorectal adenocarcinoma (35). Western blot analysis revealed that a high expression of NMT2 in human colorectal tumor tissues compared to normal tissues (Fig. 1A). The quantitative analysis demonstrated a significant increase in NMT2 expression in colorectal cancer tissues compared to normal mucosa (Fig. 1B). Interestingly, we observed a high expression of NMT2 in polyps. We demonstrate for the first time a higher expression of NMT2 in colorectal adenocarcinoma patients and HCCLs. These results indicate that the NMT2 gene is upregulated during molecular events that take place during the malignant formation of colon tissues. Higher expression of NMT2 was reported in rat hepatoma cells by dioxin toxicity and the inducible level of NMT2 was a direct consequence of Ah receptor activation (36). This observation indicates that NMT2 may not be involved in the myristoylation of $pp60^{c\text{-}src}$, but may have a role in myristoylation of other proteins in colon adenocarcinomas. Further studies are needed to distinguish the regulation of NMT1 and NMT2 on the myristoylation of various proteins in colon carcinogenesis. The specific role of each form of NMT in protein myristoylation has not yet been studied.

4. Interaction of NMTI and NMT2 with proteases

Recently, the importance of calpain in the metastatic process has received great attention. Calpain-mediated proteolysis represents a major pathway of post-translational modification that influences various aspects of cell physiology including apoptosis, cell migration and cell proliferation (37-39). Calpains cause limited proteolysis of substrates, resulting in the alteration of substrate activity. PEST sequences are believed to be the intramolecular signals for rapid proteolytic degradation by *m*-calpain. Our previous study suggested that bovine cardiac NMT1 has poor PEST regions and the elimination of NMT activity by *m*-calpain proteolysis occurs *in vitro* (40). Protein-protein interactions are controlled by the

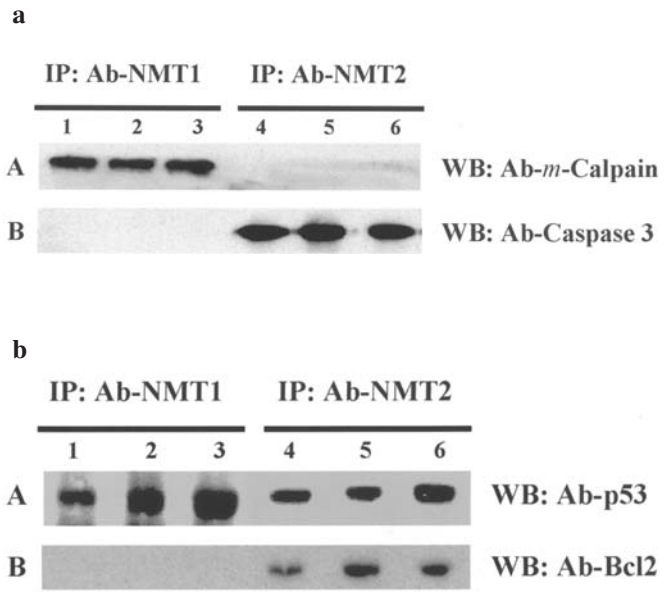

Figure 2. (a) Interaction between NMTs (NMT1 and NMT2) and proteases (*m*-calpain and caspase-3) by immunoprecipitation analysis in human colon cancer. (b) Interaction of NMTs (NMT1 and NMT2) between p53 and Bcl2 immunoprecipitation analysis in human colon cancer. Lanes 1 and 4, human colorectal normal; lanes 2 and 5, tumor; and lanes 3 and 6, HT29. For details see (35).

availability of the component proteins, which are principally controlled by the net effects of synthesis and degradation of proteins. The calpain and ubiquitin-proteasome pathways function as the major proteolytic systems responsible for the regulated degradation of various proteins (41,42). Numerous lines of evidence demonstrate that calpains, a family of Ca^{2+}-dependent cysteine protease, are involved in oncotic cell death in a variety of models (42). Recently, we reported that the activity and protein expression of *m*-calpain was significantly higher in colorectal adenocarcinomas than in normal samples (43).

We examined the interaction of both forms of NMT with *m*-calpain and caspase-3 in human colorectal normal and adenocarcinoma tissues and in HT29 by immunoprecipitation analysis (35). We observed the interaction of NMT1 with *m*-calpain in normal, tumor and HT29 samples (Fig. 2aA). Furthermore, the immunoprecipitation analysis of *m*-calpain using NMT2 antibody revealed no interaction between NMT2 and *m*-calpain (Fig. 2aA). The data revealed that *m*-calpain could interact with NMT1, but not with NMT2. This result indicates that both forms of NMT may regulate differently in cellular signaling. Cross-talk between calpain and caspase proteolytic systems has complicated efforts to determine their distinct roles in apoptotic cell death. It has been observed that calpastatin overexpression decreased calpain activation, increased caspase-3-like activity, and accelerated the appearance of apoptotic nuclear morphology (44).

The involvement of calpains and caspases in pathological conditions are unclear. However, the endogenous calpain inhibitor, calpsastatin, was degraded not by calpains but by caspases during apoptosis (45). It may be possible that caspase-3 might indirectly activate calpain via calpastatin degradation. To study the protein-protein interaction of NMTs with caspase-3, we examined immunoprecipitation analysis of caspase-3 with NMT1 and NMT2 antibodies using human normal, tumor and HT29 samples (Fig. 2aB) (35). We observed that the NMT2 interacts with caspase-3 in normal and cancerous samples whereas, NMT1 failed to interact with caspase-3 (Fig. 2aB). These data revealed that NMTs may be involved in the calpain/caspase-mediated pathway during the development of cancer. It is worth noting the difference in the interaction of the two forms of NMT with *m*-calpain and caspase-3. NMT1 was able to interact with *m*-calpain, but not with NMT2, whereas, NMT2 could interact with caspase-3 but not with *m*-calpain. It is plausible that a differential regulation exists for NMT1 and NMT2 by *m*-calpain and caspase-3.

5. Interaction of NMTs between p53 and Bcl2

Mutations in the p53 gene are among the most common genetic disorders in human cancer, including those originating in the breast, colon, lung and liver (46). Increased expressions of NMT in p53 mutant cases suggest that wild-type p53 may have a negative regulatory effect on NMT gene expression (31). It is critical to identify the pathways responsible for the activation and suppression of p53 activity in cancerous cells. The immunoprecipitation analysis of p53 with NMT1 and NMT2 antibodies revealed the interaction of p53 with NMT1 and NMT2 in human normal, tumor and HT29 samples (Fig. 2bA) (35). Interaction of p53 with NMT1 was more intense in human colorectal adenocarcinoma than in normal mucosa. Similarly, the interaction of p53 with NMT1 in HT29 cells was more significant (Fig. 2bA). We observed the interaction of p53 with NMT2 in human normal and tumor tissues extracts and in HT29. While, no change in intensity of interaction between NMT2 and p53 was observed across normal and cancerous tissues, a slightly higher interaction occurred in HT29 cells (Fig. 2bA). These data suggest that NMTs may be involved in the p53 pathway during cancer development.

Bcl2 overexpression leading to inhibition of cell death signaling has been observed as a relatively early event in colorectal cancer development. Several studies of colorectal adenocarcinomas have detected expression of Bcl2 protein using immunohistochemistry (28,47-52). We examined the interaction of Bcl2 with NMT1 and NMT2 by immunoprecipitation analysis in human normal, tumor and HT29 samples (Fig. 2bB) (35). NMT1 failed to interact with Bcl2 in any of the samples tested (Fig. 2bB), whereas Bcl2 interacted with NMT2 (Fig. 2bB). These data suggest that Bcl2 associates with NMT2, but not with NMT1 (35).

Despite the massive amount of knowledge that has accumulated about p53, there is still much to learn about its role in tumor suppression. Tumors with increased expression of NMT and p53 were associated with poor clinical outcomes as evidenced by their mean survival times (31). Selvakumar *et al* (35) showed a strong interaction between NMT1 and p53 in colon cancer tissues and in HT29. However, the interaction of NMT2 with p53 was similar in cancerous and normal mucosa. It is critical to identify the pathways responsible for suppression of apoptosis by NMT1 and NMT2 in colon cancer. Bcl2 suppresses apoptosis induced by a wide variety of stimuli in multiple cell types. Our present study sheds light

on the interaction of two forms of NMT with Bcl2. The interesting observation was that Bcl2 interacts with NMT2, whereas this apoptotic factor was unable to interact with NMT1 (35). Further studies are needed to explain the role of NMT1 and NMT2 in the regulation of apoptosis in colon carcinoma.

6. Conclusion

We demonstrated the overexpression of NMT2 in colorectal cancer tissues as well as in HCCLs employing Western blot analysis. Furthermore, we observed the interaction of different forms of NMT (NMT1 and NMT2) with different cell signalling molecules in colon cancer. NMT1 and NMT2 interact differently with *m*-calpain and caspase-3. Further work is required to rule out the possibility that myristoylation is regulated by *m*-calpain and caspase-3 using particular protease inhibitors. Strong protein-protein interaction of NMT1 with p53 was observed in colon cancer tissues and HT29. Currently, we are investigating the specific role of NMT1 and NMT2 by RNAi studies on myristoylation of proteins and their involvement in the regulation of apoptosis.

Acknowledgements

This work is supported by the Canadian Institutes of Health Research (Grant No. MOP-36484) Canada. P. Selvakumar was a recipient of Postdoctoral Fellowship from the Saskatchewan Health Research Foundation, Saskatoon, Canada.

References

1. Wilcox C, Hu JS and Olson EN: Acylation of proteins with myristic acid occurs cotranslationally. Science 238: 1275-1278, 1987.
2. Selvakumar P, Lakshmikuttyamma A, Shrivastav A, Das SB, Dimmock JR and Sharma RK: Potential role of *N*-myristoyltransferase in cancer. Prog Lipid Res doi:10.1016/j.plipres.2006.05.002, 2006.
3. Selvakumar P, Lakshmikuttyamma A, Charavaryamath C, Singh B, Tuchek J and Sharma RK: Expression of myristoyltransferase and its interacting proteins in epilepsy. Biochem Biophys Res Commun 335: 1132-1139, 2005.
4. Selvakumar P, Pasha MK, Ashakumary L, Dimmock JR and Sharma RK: Myristoyl-CoA:protein N-myristoyltransferase: A novel molecular approach for cancer therapy. Int J Mol Med 10: 493-500, 2002.
5. Farazi TA, Waksman G and Gordon JI: The biology and enzymology of protein N-myristoylation. J Biol Chem 276: 39501-39504, 2002.
6. Rajala RV, Datla RS, Moyana TN, Kakkar R, Carlsen SA and Sharma RK: N-myristoyltransferase. Mol Cell Biochem 204: 135-155, 2000.
7. Resh MD: Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochim Biophys Acta 1451: 1-16, 1999.
8. Boutin JA: Myristoylation. Cell Signal 9: 15-35, 1997.
9. King MJ and Sharma RK: Demonstration of multiple forms of bovine brain myristoyl CoA:protein N-myristoyl transferase. Mol Cell Biochem 113: 77-81, 1992.
10. McIlhinney RA, McGlone K and Willis AC: Purification and partial sequencing of myristoyl-CoA:protein N-myristoyltransferase from bovine brain. Biochem J 290: 405-410, 1993.
11. Glover CJ and Felsted RL: Identification and characterization of multiple forms of bovine brain N-myristoyltransferase. J Biol Chem 270: 23226-23233, 1995.
12. Giang DK and Cravatt BF: A second mammalian N-myristoyltransferase. J Biol Chem 273: 6595-6598, 1998.
13. Rundle DR, Rajala RV and Anderson RE: Characterization of Type I and Type II myristoyl-CoA:protein N-myristoyltransferases with the Acyl-CoAs found on heterogeneously acylated retinal proteins. Exp Eye Res 75: 87-97, 2002.
14. Wiegand RC, Carr C, Minnerly JC, Pauley AM, Carron CP, Langner CA, Duronio RJ and Gordon JI: The *Candida albicans* myristoyl-CoA:protein N-myristoyltransferase gene. Isolation and expression in *Saccharomyces cerevisiae* and *Escherichia coli*. J Biol Chem 267: 8591-8598, 1992.
15. Lodge JK, Johnson RL, Weinberg RA and Gordon JI: Comparison of myristoyl-CoA:protein N-myristoyltransferases from three pathogenic fungi: *Cryptococcus neoformans, Histoplasma capsulatum*, and *Candida albicans*. J Biol Chem 269: 2996-3009, 1994.
16. Raju RV, Anderson JW, Datla RS and Sharma RK: Molecular cloning and biochemical characterization of bovine spleen myristoyl CoA:protein N-myristoyltransferase. Arch Biochem Biophys 348: 134-142, 1997.
17. Qi Q, Rajala RV, Anderson W, Jiang C, Rozwadowski K, Selvaraj G, Sharma R and Datla R: Molecular cloning, genomic organization, and biochemical characterization of myristoyl-CoA:protein N-myristoyltransferase from *Arabidopsis thaliana*. J Biol Chem 275: 9673-9683, 2000.
18. Raju RV, Kalra J and Sharma RK: Purification and properties of bovine spleen N-myristoyl-CoA protein:N-myristoyltransferase. J Biol Chem 269: 12080-12083, 1994.
19. Ntwasa M, Aapies S, Schiffmann DA and Gay NJ: *Drosophila* embryos lacking N-myristoyltransferase have multiple developmental defects. Exp Cell Res 262: 134-144, 2001.
20. Zha J, Weiler S, Oh KJ, Wei MC and Korsmeyer SJ: Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. Science 290: 1761-1765, 2000.
21. Utsumi T, Sakurai N, Nakano K and Ishisaka R: C-terminal 15 kDa fragment of cytoskeletal actin is posttranslationally N-myristoylated upon caspase-mediated cleavage and targeted to mitochondria. FEBS Lett 539: 37-44, 2003.
22. Jackson R and Hunter T: Role of methionine in the initiation of haemoglobin synthesis. Nature 227: 672-676, 1970.
23. Selvakumar P, Lakshmikuttyamma A, Kanthan R, Kanthan SC, Dimmock JR and Sharma RK: High expression of methionine aminopeptidase 2 in human colorectal adenocarcinomas. Clin Cancer Res 10: 2771-2775, 2004.
24. Selvakumar P, Lakshmikuttyamma A, Lawman Z, Bonham K, Dimmock JR and Sharma RK: Expression of methionine aminopeptidase 2, N-myristoyltransferase, and N-myristoyltransferase inhibitor protein 71 in HT29. Biochem Biophys Res Commun 322: 1012-1017, 2004.
25. Park J, Meisler AI and Cartwright CA: c-Yes tyrosine kinase activity in human colon carcinoma. Oncogene 8: 2627-2635, 1993.
26. Bolen JB, Veillette A, Schwartz AM, DeSeau V and Rosen N: Activation of $pp60^{c\text{-}src}$ protein kinase activity in human colon carcinoma. Proc Natl Acad Sci USA 84: 2251-2255, 1987.
27. Biscardi JS, Ishizawar RC, Silva CM and Parsons SJ: Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer. Breast Cancer Res 2: 203-210, 2000.
28. Bosari S, Moneghini L, Graziani D, Lee AK, Murray JJ, Coggi G and Viale G: Bcl-2 oncoprotein in colorectal hyperplastic polyps, adenomas, and adenocarcinomas. Hum Pathol 26: 534-540, 1995.
29. Magnuson BA, Raju RV, Moyana TN and Sharma RK: Increased N-myristoyltransferase activity observed in rat and human colonic tumors. J Natl Cancer Inst 87: 1630-1635, 1995.
30. Raju RV, Moyana TN and Sharma RK: N-Myristoyltransferase overexpression in human colorectal adenocarcinomas. Exp Cell Res 235: 145-154, 1997.
31. Rajala RV, Radhi JM, Kakkar R, Datla RS and Sharma RK: Increased expression of N-myristoyltransferase in gallbladder carcinomas. Cancer 88: 1992-1999, 2000.
32. Lu Y, Selvakumar P, Ali K, Shrivastav A, Bajaj G, Resch L, Griebel R, Fourney D, Meguro K and Sharma RK: Expression of N-myristoyltransferase in human brain tumors. Neurochem Res 30: 9-13, 2005.
33. King MJ and Sharma RK: Identification, purification and characterization of a membrane-associated N-myristoyltransferase inhibitor protein from bovine brain. Biochem J 291: 635-639, 1993.

34. Selvakumar P, Lakshmikuttyamma A, Pasha MK, King MJ, Olson DJ, Mori S, Ross AR, Hayashi K, Dimmock JR and Sharma RK: N-myristoyltransferase inhibitor protein is homologous to heat shock cognate protein 70. J Cell Biochem 92: 573-578, 2004.
35. Selvakumar P, Smith-Windsor E, Bonham K and Sharma RK: N-myristoyltransferase 2 expression in human colon cancer: cross-talk between the calpain and caspase system. FEBS Lett 580: 2021-2026, 2006.
36. Kolluri SK, Balduf C, Hofmann M and Gottlicher M: Novel target genes of the Ah (dioxin) receptor: transcriptional induction of N-myristoyltransferase 2. Cancer Res 61: 8534-8539, 2001.
37. Porter AG and Janicke RU: Emerging roles of caspase-3 in apoptosis. Cell Death Differ 6: 99-104, 1999.
38. Carragher NO, Fincham VJ, Riley D and Frame MC: Cleavage of focal adhesion kinase by different proteases during SRC-regulated transformation and apoptosis. Distinct roles for calpain and caspases. J Biol Chem 276: 4270-4275, 2001.
39. Huttenlocher A, Palecek SP, Lu Q, Zhang W, Mellgren RL, Lauffenburger DA, Ginsberg MH and Horwitz AF: Regulation of cell migration by the calcium-dependent protease calpain. J Biol Chem 272: 32719-32722, 1997.
40. Raju RV, Kakkar R, Datla RS, Radhi J and Sharma RK: Myristoyl-coA:protein N-myristoyltransferase from bovine cardiac muscle: molecular cloning, kinetic analysis, and *in vitro* proteolytic cleavage by m-calpain. Exp Cell Res 241: 23-35, 1998.
41. Rajkumar SV, Richardson PG, Hideshima T and Anderson KC: Proteasome inhibition as a novel therapeutic target in human cancer. J Clin Oncol 23: 630-639, 2005.
42. Carragher NO and Frame MC: Calpain: a role in cell transformation and migration. Int J Biochem Cell Biol 34: 1539-1543, 2002.
43. Lakshmikuttyamma A, Selvakumar P, Kanthan R, Kanthan SC and Sharma RK: Overexpression of *m*-calpain in human colorectal adenocarcinomas. Cancer Epidemiol Biomarkers Prev 13: 1604-1609, 2004.
44. Neumar RW, Xu YA, Gada H, Guttmann RP and Siman R: Cross-talk between calpain and caspase proteolytic systems during neuronal apoptosis. J Biol Chem 278: 14162-14167, 2003.
45. Wang KK, Posmantur R, Nadimpalli R, Nath R, Mohan P, Nixon RA, Talanian RV, Keegan M, Herzog L and Allen H: Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis. Arch Biochem Biophys 356: 187-196, 1998.
46. Fearon ER and Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 61: 759-767, 1990.
47. Hague A, Moorghen M, Hicks D, Chapman M and Paraskeva C: Bcl-2 expression in human colorectal adenomas and carcinomas. Oncogene 9: 3367-3370, 1994.
48. Sinicrope FA, Ruan SB, Cleary KR, Stephens LC, Lee JJ and Levin B: Bcl-2 and p53 oncoprotein expression during colorectal tumorigenesis. Cancer Res 55: 237-241, 1995.
49. Ofner D, Riehemann K, Maier H, Riedmann B, Nehoda H, Totsch M, Bocker W, Jasani B and Schmid KW: Immunohistochemically detectable Bcl-2 expression in colorectal carcinoma: correlation with tumor stage and patient survival. Brit J Cancer 72: 981-985, 1995.
50. Watson AJM, Merritt AJ, Jones LS, Askew JN, Anderson E, Becciolini A, Balzi M, Potten CS and Hickman JA: Evidence of reciprocity of bcl-2 and p53 expression in human colorectal adenomas and carcinomas. Br J Cancer 73: 889-895, 1996.
51. Baretton GB, Diebold J, Christoforis G, Vogt M, Muller C, Dopfer K, Schneiderbanger K, Schmidt M and Lohrs U: Apoptosis and immunohistochemical bcl-2 expression in colorectal adenomas and carcinomas. Cancer 77: 255-264, 1996.
52. Flohil CC, Janssen PA and Bosman FT: Expression of Bcl-2 protein in hyperplastic polyps, adenomas, and carcinomas of the colon. J Pathol 178: 393-397, 1996.