# N-myristoyltransferase: A Novel Molecular Marker for Colorectal Adenomatous Polyps and Cancer

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## Background

Protein N-myristoylation refers to the covalent attachment of myristate, a 14-carbon saturated fatty acid, to the N-terminal glycine residue of proteins. The recent discovery that lysine residues of proteins can also be myristoylated further emphasized the significance of myristoylation. Existing as two isoforms that share 77% homology, myristoylation of proteins is catalyzed by an enzyme N-myristoyltransferase (NMT) (1). Many oncoproteins such as c-Abl, ARF, cAMP dependent protein kinase, and various tyrosine kinases (pp60src, pp60Yes, pp56lck, pp59fyn/syn) that are crucial for the onset and progression of cancer are myristoylated (2).

There are various pathways like mTOR, PI3K-Akt, Ras/Raf/MEK/ERK, Notch, and others, which play a significant role in the proliferation of neuroendocrine cancers (3-5). The dysregulation of these pathways presents themselves as molecular signatures that are potential therapeutic targets and diagnostic markers in managing neuroendocrine tumors. We recently demonstrated that the expression of NMT1 increased with rapamycin treatment over the period with a concomitant decrease in mTOR phosphorylation (6).

### Significance to NET-GI

We observed overexpression of NMT2 in PBMC of subjects with colorectal adenomatous polyps and cancers, which agrees with previously reported observation of high expression of NMT2 in colorectal polyps and cancer tissues. Our results from breast cancer and colorectal cancer (CRC) studies suggest that NMT is a crucial regulator of onco-pathways, especially PI3K/mTOR pathway. The expression profile of NMT in the neuroendocrine tumor in the gastrointestinal tract (NET-GI) could result in the identification of a novel therapeutic targets. The inhibitors of NMT in clinical trials could potentially benefit in the management of NET-GI. In this prospective study, we demonstrated that NMT2 is a molecular marker for detecting colorectal adenomatous polyps and cancer.

## Objective

To evaluate the performance of NMT2 in detection of colorectal adenomatous polyps and cancer by determining the sensitivity and specificity.

## Material and Method

### Experimental Approach

*Ethics*

The ethics approval for the study was obtained from the Human Research Ethics Board, University of Manitoba.

*Recruitment*

A total of 74 subjects were recruited prospectively (no indication of disease (NED) with a normal lower gastrointestinal tract endoscopy = 24; adenomatous polyps (AP) = 19; non-adenomatous polyps (NAP) = 12; CRC= 15); 4 of the subjects had polyps confirmed by colonoscopy.

### Experimental Approach

*Immunohistochemistry (IHC)*

Cytospin slides of PBMCs were prepared in duplicate using cytospin centrifuge and sent for IHC at the Manitoba Tumor Bank in CancerCare Manitoba, Winnipeg. The PBMC Cytospin slides were stained for NMT2 on a Ventanna autostainer. An H-scores derived from assessing both staining intensity (scale 0-3) and the percentage of positive cells (0-100%) was given to each sample. These two scores were multiplied to generate an H-score of 0-300 (2).

## Results

We report for the first time that N-myristoyltransferase 2 is significantly upregulated in peripheral blood mononuclear cells (PBMC) of subjects with colorectal adenomatous polyps and cancer compared to individuals with non-adenomatous polyps or no evidence of disease. Collectively, our findings provide evidence that NMT2 could be a promising biomarker for the detection of pre-malignant adenomatous polyps as well as CRC and can serve as a potential screening test (7).

### Performance of NMT-2 H-scoring in identification of CRC subjects

The performance of NMT2 in detecting CRC was evaluated by setting our primary endpoint to assess the level of NMT2 expression in subjects with CRC (n=15) compared to subjects without CRC (n=59). Table 1 lists the clinicopathologic characteristics of these two groups; subjects with CRC were significantly older and more often had history of non-CRC cancers (Table 1). In the univariate analysis, subjects with CRC had significantly elevated expression of NMT2 with high H scores (median of 180) compared to subjects without CRC (median 90), Figure 1A and 1B ($p < 0.001$).

In a multivariate analysis accounting for the effects of age and other cancers, this difference remained significant. The ROC curve was constructed based on these data and had an area under the curve (AUC) of 0.83, which increased upon adjusting for age and presence of cancers other than CRC to 0.86 (Figure 1C). ROC curve is the plot of sensitivity vs. 1-specifity.

**Table 1.** Clinicopathologic characteristics of participants (n=74).

| Subject characteristics | CRC (n=15) | Non-CRC-(n=59) | P value |
|---|---|---|---|
| **Median age (IQR)** | 68 (61-73) | 60 (49-67) | **0.011** |
| **Men, n (%)** | 5 (33.3) | 29 (49.2) | 0.272 |
| **Other cancers of non-CRC origin, n (%)*** | 4 (26.7) | 0 (0) | **<0.001** |
| **Family history of polyps and/or cancer, n (%)** | 8 (53.3) | 17 (28.8) | 0.073 |
| **Diverticulosis, n (%)** | 2 (13.3) | 17 (28.8) | 0.220 |
| **Hemorrhoids, n (%)** | 4 (26.7) | 19 (32.2) | 0.679 |
| **Inflammatory bowel conditions, n (%), of which:** | 2 (13.3) | 10 (16.9) | 0.734 |
| UC/CD | 1 (6.7) | 6 (10.2) | |
| Other** | 1 (6.7) | 4 (6.8) | |
| **Lynch syndrome, n (%)** | 0 (0) | 2 (3.4) | 0.470 |

Ulcerative colitis (UC), Crohn's disease (CD).
*Four individuals had tumors present in the breast and liver, pancreas, liver and lungs, prostate, kidney.
**Ileocecal valve ulcer, patchy inflammation in rectum, mild-moderate colitis.

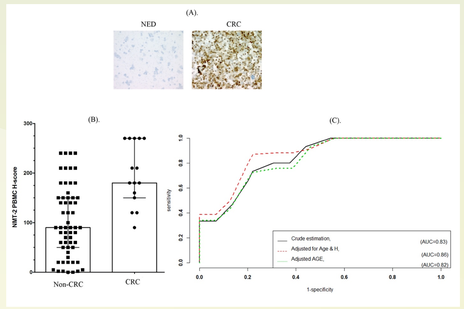


**Figure 1: Differential expression of NMT2 as determined by IHC in PBMC of CRC patients compared to subjects with no CRC.**
(A). PBMC cytospin slides were stained for NMT2 using polyclonal anti-NMT2 antibody by IHC. NMT2 expression in PBMC from subjects with no evidence of disease (NED), subjects with non-adenomatous polyps (NAP), subjects with colorectal adenomatous polyps (AP) and cancer (CRC). (B). Histogram showing the "H" score from subjects with CRC (n=15) and non-CRC (n= 59) subjects, bars of the histogram represent the mean "H" scores and the error bars represents the standard deviation between them. (C). ROC curve analysis was performed to determine the performance of NMT2 in differentiating CRC from subjects without CRC. When adjusted for the age, the accuracy of NMT2 in differentiating CRC is 86%.

### Performance of NMT2 in the Detection of Colorectal Adenomatous Polyps and Cancer

We observed that the expression of NMT2 was highly elevated in PBMC of several cases with non-CRC, therefore we further classified the non-CRC group into three clinically relevant sub-groups, NED, NAP and AP. The subjects with colorectal AP had significantly higher H score compared to subjects with NED and NAP (Figure 3A). In subjects with NED, NMT2 expression in PBMC ranged from negative to weak positivity except for 4 cases. The H score ranged from 0-240 with an average score of 70.9.

In cases where the polyps were characterized as NAP, the H score ranged from 2-210 with an average score of 70.1. In contrast, CRC patients and subjects with AP displayed strong NMT2 staining and high percentage of positive cells. The H score for colorectal AP and cancer ranged from 90-240 and 90-270, respectively with an average score of 162.6 for AP and 196.7 for CRC (Table 1). In the univariate analysis, compared to the NED, subjects with CRC and AP had significantly elevated NMT-2 H scores (median values 195 and 150, respectively, $p<0.001$ for both). The median H-score of subjects with NAP was 1.25 fold higher, but was not significant than the subjects with NED, while significantly lower than that seen in subjects with CRC and AP, 2.2-fold ($p=0.002$), and 1.9-fold ($p=0.017$), respectively. The ROC curve showed a high AUC (AUC=0.88, 95% CI: 78.7 – 94.9%; Figure 2 D, Table 2). Furthermore, the positive predictive value (PPV) and negative predictive (NPV) value were calculated at each cut-off point for NMT2 expression. At the optimal cutoff, which maximizes the average of the sensitivity and specificity, the overall probability of correct classification was optimized as well. Various cutoff for H scores with respect to sensitivity and specificity is provided in table 2. An H score of 120 represented a high sensitivity (93.94%) and specificity (83.78%) in identifying both AP and CRC compared to NED and NAP.

## Acknowledgement

SVS acknowledge the support from CSB Manitoba, Canada.

**Table 2**: Sensitivity and Specificity of NMT2 in detecting CRC/adenomatous polyps.

| Cutoff-point | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|
| ≥ 0 | 100 | 0 | 47.14 | N/A |
| ≥ 2 | 100 | 5.41 | 49.53 | 100 |
| ≥ 5 | 100 | 10.81 | 49.99 | 100 |
| ≥ 10 | 100 | 16.22 | 51.56 | 100 |
| ≥ 20 | 100 | 21.62 | 53.22 | 100 |
| ≥ 30 | 100 | 35.14 | 57.89 | 100 |
| ≥ 40 | 100 | 37.84 | 58.96 | 100 |
| ≥ 50 | 100 | 40.54 | 60 | 100 |
| ≥ 60 | 100 | 48.65 | 63.91 | 100 |
| ≥ 70 | 100 | 59.46 | 68.75 | 100 |
| ≥ 80 | 100 | 64.86 | 71.73 | 100 |
| ≥ 90 | 100 | 70.27 | 74.99 | 100 |
| ≥ 100 | 93.94 | 81.08 | 81.58 | 93.75 |
| ≥ 120 | 93.94 | 83.78 | 83.77 | 93.93 |
| ≥ 140 | 78.79 | 83.78 | 81.24 | 81.58 |
| ≥ 150 | 66.67 | 83.78 | 78.56 | 73.81 |
| ≥ 160 | 54.55 | 86.49 | 78.27 | 68.09 |
| ≥ 180 | 48.48 | 86.49 | 76.56 | 65.75 |
| ≥ 210 | 30.30 | 89.19 | 71.42 | 58.92 |
| ≥ 240 | 24.24 | 97.30 | 88.89 | 59.01 |
| ≥ 270 | 15.15 | 100 | 100 | 56.92 |
| > 270 | 0 | 100 | N/A | 52.05 |

The table provides the PPV and NPV at each NMT2 cut-off. At the optimal cut-off, which maximizes the average of the sensitivity and specificity, the overall probability of correct classification was optimized as well. It is a coincidence that PPV = specificity & NPV = sensitivity at the optimal cut-off point.

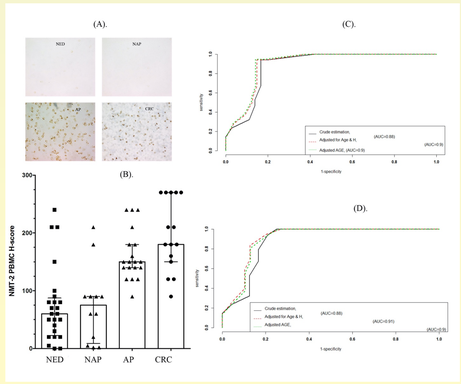


**Figure 2: NMT2 levels are augmented in PBMC obtained from colorectal adenomatous polyps and cancers.**
(A). PBMC cytospin slides were stained for NMT2 using polyclonal anti-NMT2 antibody by IHC. NMT2 expression in PBMC from subjects with no evidence of disease (NED), subjects with non-adenomatous polyps (NAP), subjects with colorectal adenomatous polyps (AP) and cancer (CRC). (B). H scores of NMT2 expression in PBMC of NED (n=24), NAP (n=12), AP (n=19) and CRC (n=15). Bars of the histogram represents the mean "H" scores within each group and the error bars represents the standard deviation between them. There is no significant difference between NED and NAP ($P =$(C). ROC curve of CRC+AP vs. NED+NAP and (D). CRC+AP vs. NED.

## Conclusion

Here we longitudinally examined the utility of blood NMT expression as a biomarker for colorectal cancer. Our analysis demonstrated that the NMT2 H-scores perform best for distinguishing CRC/AP from NED/NAP. The levels of NMT-2 protein in PBMC appear to gradually increase along with the natural progression of polyp development from non-adenomatous to adenomatous to CRC. In our future study, we plan to assess the NMT1/NMT2 expression in subjects with NET and investigate its utility as a diagnostic marker and therapeutic target.

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