Methods of quantifying DNA methylation and other modified DNA bases in gastric cancer cell lines using LC-MS/MS

Wenxia Luo [1], Olufola Ige [1], and Ted M Lakowski [1]

1, College of Pharmacy, Pharmaceutical Analysis Laboratory, University of Manitoba, Winnipeg, Manitoba, Canada;

UM | Rady Faculty of Health Sciences

ABSTRACT

Introduction: Cytosine methylation (mdC) is an epigenetic modification to DNA that controls gene expression, and several new DNA modifications have been discovered. Aberrant deposition of promoter mdC is associated with gastric cancer. However, no method has been developed to quantify all DNA modifications from cells and such methods are needed to develop metabolomic biomarker screens for cancer and to evaluate new treatments for cancer. We developed and validated an LC-MS/MS method to quantify 5-methylcytosine (5-mdC), 5-hydroxymethylcytosine (5-hmdC), 5-formylcytosine (5-fdC), 5-carboxycytosine (5-cadC), methyl adenosine (mdA) mdA, and the 4 canonical DNA bases from hydrolyzed genomic DNA extracted from cultured cells. *Methods:* The methods were developed and validated using synthetic 20mer dsDNA oligonucleotides each containing a single modified nucleotide. The oligonucleotides were enzymatically hydrolyzed into nucleosides and the free canonical and modified bases quantified using LC-MS/MS. The method was evaluated using hydrolyzed genomic DNA extracted from gastric cancer cell lines. *Results:* The methods were capable of quantitative recovery of the canonical and modified DNA bases from the synthetic oligonucleotides and accuracy precision and low limit of quantification were determined. We detected elevated 5-mdC, mdA in gastric cell lines HEK 293, SNU 719, AGS and MKN 45 treated with DNA methyltransferase inhibitors. *Conclusions:* The LC-MS/MS methods can quantify the total amounts of the modified bases on DNA isolated from cancer cell lines. This method will be further developed to generate metabolomic biomarker screens and to test DNA methyltransferase inhibitors on cancer cell lines.

INTRODUCTION

In Canada, it is estimated that 1 in 98 Canadians will develop gastric cancer in their lifetime and the fact that patients with gastric cancer exhibit high incidence, metastasis, and mortality and a low early diagnosis, and 5-year survival rate. Cytosine methylation (mdC) is a known epigenetic modification to DNA that controls gene expression. Abnormal mdC deposition is involved in gastric cancer, particularly hypermethylation of the p16 promoter reduces its expression and results in gastric cancer. Several new DNA modifications have been discovered as well, but further studies are needed to demonstrate their functional importance. Methods are needed to quantify all DNA modifications from cells to develop metabolomic biomarker screens for cancer and to evaluate new treatments for cancer. We developed and validated an LC-MS/MS method to quantify mdC, 5-hydroxymethylcytosine (5-hmdC), 5-formylcytosine (5-fdC), 5-carboxycytosine (5-cadC), methyl adenosine (mdA) and the 4 canonical DNA bases from hydrolyzed genomic DNA, extracted from cultured cells. We also utilize these methods to evaluate different gastric cells lines treated with DNMT inhibitors.

OBJECTIVES

- To develop and validate a LC-MS/MS method for quantifying DNA methylation and other modified DNA bases in gastric cancer cell lines
- To evaluate different modified DNA bases in gastric cancel lines treated with different DNMT inhibitors compared with p16 expression.

MATERIALS AND METHODS

The methods were developed and validated using synthetic 20mer dsDNA oligonucleotides, each containing a single modified nucleotide. The oligonucleotides were enzymatically hydrolyzed into nucleosides. The method was evaluated using hydrolyzed genomic DNA extracted from gastric cancer cell line.

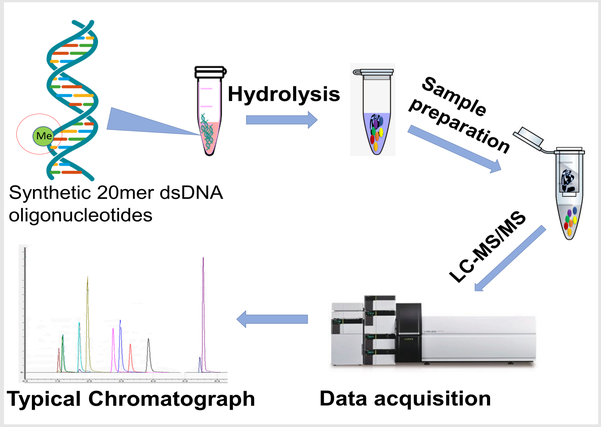

Figure 1. The flow of optimization and validation for LC-MS/MS methods

LC-MS/MS Method optimization and validation

- LC condition and MS condition
- Methods Validation

✓ Performed according to the FDA guideline

✓ Selectivity, sensitivity, linearity, accuracy and precision, recovery, matrix effect

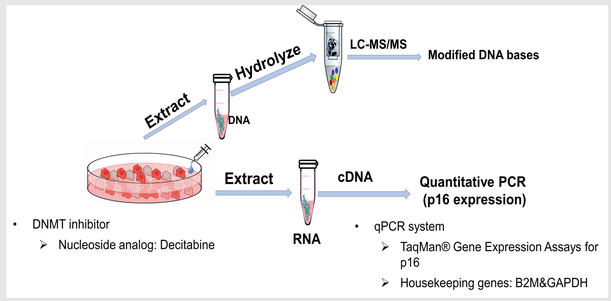

Figure 2. The schematic diagram of evaluation for genomic DNA extracted from treated gastric cancer cell line compared with p16 expression

RESULT

Linearity

Analyte	Calibration Curve			
	LLOQ (nM)	ULOQ (nM)	Typical Equation	R^2
mdC	2	800	y=0.002293x+0.996	0.9968
mdA	0.2	80	y=0.012664x+0.993	0.9936
hmdC	0.4	160	y=0.014453x+0.001	0.9964
fdC	0.8	320	y=0.011314x+0.997	0.9977
cadC	0.8	320	y=0.001375x+0.991	0.9920
dC	2X10^3	2X10^5	y=0.022123x+0.997	0.9973
dA	2X10^3	2X10^5	y=0.005180x+0.998	0.9989
dG	2X10^3	2X10^5	y=0.004655x+0.988	0.9883
dT	2X10^3	2X10^5	y=0.004371x+0.998	0.9981

Table 1 Linearity of 9 DNA bases in cell samples

Accuracy and precision (n=3 with 6 replicates per batch)

- Intra- and inter-batch accuracy (RE) <12.8%
- Intra- and inter-batch precision (bias) <14.5%

Matrix effect< 9.2%

The methods were capable of quantitative recovery of the above modified DNA bases from the synthetic oligonucleotides and accuracy precision and low limit of quantitation were determined.

Application

- **Evaluation of DNA Hydrolysis recovery**

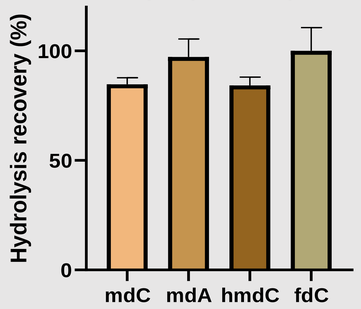

Figure 4. hydrolysis result for modified DNA bases

- **Evaluation of DNA modifications and p16 expression for DNA methyltransferase inhibitors on cancer cell lines**

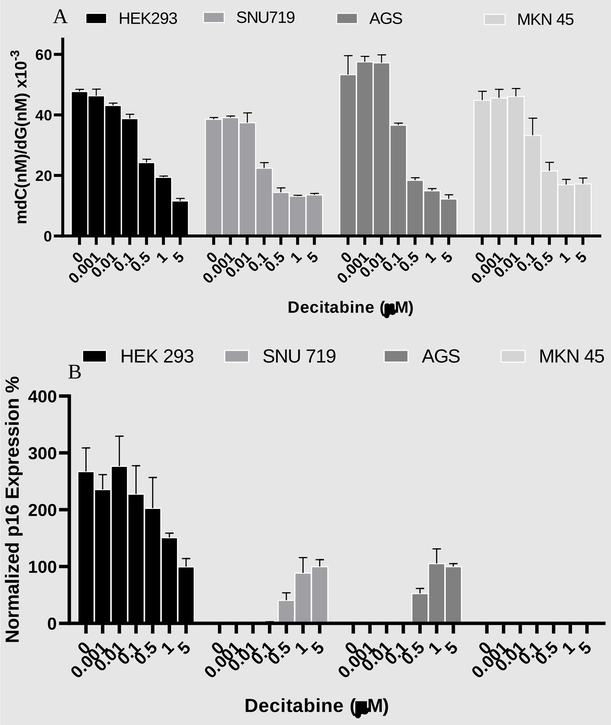

Figure 5. Total methylation status (A) and p16 expressions (B) of cell lines (HEK 293, SNU 719,AGS and MKN45) treated with decitabine in 72 hours (N = 3).

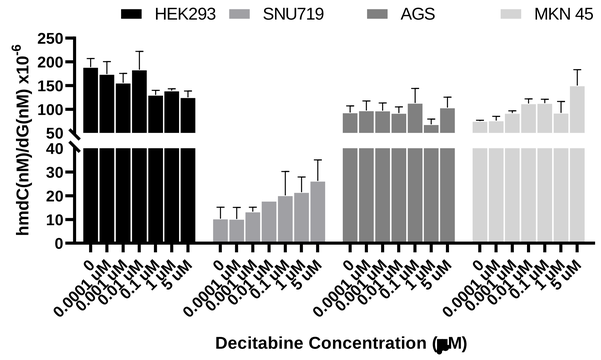

Figure 6. hmdC status of cell lines (HEK 293, SNU 719,AGS and MKN45) treated with decitabine in 72 hours (N = 3).

CONCLUSIONS

The LC-MS/MS methods have adequate sensitivity, acceptable precision and accuracy, which can quantify the total amounts of the above modified bases on DNA isolated from cells. These methods are used to test DNA methyltransferase inhibitors on cancer cell lines and will be further developed to generate metabolomic biomarker screens on cancer cell lines. It shows that decitabine can decrease the total methylation status for all the cell lines. The reduction of DNA methylation status is consistent with p16 expression alterations shown for SNU 719 and AGS cell lines. However, in MKN 45, even in the highest concentration of decitabine, there is no expression of p16, which demonstrated that hypermethylation of p16 promoter region may not the main cause of the lack of p16 expression in MKN 45 cells. In SNU 719, the hmdC increases with decitabine concentration, but there is no significant change in the other cell lines. Based on our result, SNU 719 is a DNMT inhibitor sensitive cell line and is an appropriate model for the link between p16 expression and CpG methylation in humans.

FUTURE DIRECTIONS

- Investigate more DNMT inhibitors such as RG 108, DC 517 by using our LC-MS/MS method
- Elevate the change histone modification of these cell lines such as HEK 293, SNU 719, AGS and MKN45 treated with DNMT inhibitors
- Combine and analyze the data for DNA modificaton and histone modification in different gastric cancer cell lines with DNMT inhibitors

REFERENCE

1. X. Wu and Y. Zhang, Nat. Rev. Genet., vol. 18, no. 9, pp. 517–534, 2017.
2. Q. Xie et al., Cell, vol. 175, no. 5, pp. 1228-1243.e20, 2018.
3. M. R. Abbaszadegan et al., World J. Gastroenterol.,pp. 2055–2060, 2008.
4. D. He et al., Med. Oncol., vol. 32, no. 4, pp. 1–16, Apr. 2015.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge support from NSERC, CIHR and University of Manitoba Graduate Fellowship and Faculty of Graduate Studies Research Completion Scholarship (To W.L)

CIHR IRSC Canadian Institutes of Health Research | Instituts de recherche en santé du Canada

NSERC CRSNG