MTHFD2 Expression Association with Bladder Cancer and Screening of its Potential Inhibitor

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Abstract

Background

Bladder cancer is the most common urological malignancy. Genes of folate mediated 1 carbon metabolism are found to be highly up regulated in tumor cells and promotes tumor cell proliferation.

Rationale and aim of the study

The aim of the current study was to determine the expression of MTHFD2 gene and the impact of intronic SNP rs1667627 on MTHFD2 expression. Furthermore, determination of potential ligand based inhibitors against MTHFD2.

Methods & Results

Semi-quantitative expression analysis and sanger sequencing were used for this purpose. Moreover, structure based virtual ligand library screening in order to find plausible inhibitors. MTHFD2 expression was significantly increased with tumor stage progression both in low and high-grade bladder cancer. However, the relative fold change difference in low grade bladder cancer in correlation with the tumor stage progression was more dramatic. Contrary to the TCGA dataset analysis, increased MTHFD2 expression was observed in papillary bladder cancer tumor. G to A transition in the intronic variant rs1667627 SNP was determined in tumor tissues as compared to control. Virtual ligand based library screening against the three dimensional MTHFD2 protein lead to identification of a plausible inhibitor MCULE-8027924848 that displayed lower binding free energy as compared to already documented LY345899.

Conclusion

MTHFD2 might be used as low-grade bladder cancer biomarker since its expression level changes drastically with tumor progression. Further, experimental studies are required to establish the potential mode of inhibition of MCULE-8027924848 ligand.

Introduction

Bladder cancer is a malignancy of urinary tract that arises from the epithelium lining of the urinary bladder (Oliveira, Jerónimo et al. 2012). In Western countries, a prevalence of more than 1,300,000 patients has been reported with a male to female ratio of almost 3:1 (Siegel, Naishadham et al. 2012, Siegel, Miller et al. 2016). In Pakistan, it is the 4th and the 15th most common cancer among men and women primarily due to smoking based on Karachi Cancer registry (Tunio, Hashmi et al. 2011).

The metabolism of folate yields folate intermediates that fuel the one carbon metabolism. Upregulation of 1C metabolism pathway enzymes has been associated with high cancer risk. The folate metabolism occurs both in cytosol and mitochondria. Increased expression of mitochondrial folate pathway enzymes is associated with cancer development (Yang and Vousden 2016). The 1C metabolism aids in purine and thymine synthesis by activation and transfer of 1C units for this biosynthesis process (Ducker and Rabinowitz 2017). Methylenetetrahydrofolate dehydrogenase/cyclohydrolase (MTHFD2) is a bifunctional NAD(P) dependent enzyme play an important role in the mitochondrial folate mediated one carbon pathway. MTHFD2 has a role in maintaining redox homeostasis by maintaining NADPH especially in colorectal cancer cells (Ju, Lu et al. 2019). MTHFD2 is responsible for recycling of the tetra hydro-folate (THF) in the mitochondria. Increased enzymatic activity of the MTHFD2 has been found in several tumor studies (Liu, Liu et al. 2014, Tedeschi, Vazquez et al. 2015). Inhibition or depletion of MTHFD2 has shown anti-proliferative effects on cancer cells demonstrating it as a plausible therapeutic anti-cancer target (Liu, Liu et al. 2014, Gustafsson, Jemth et al. 2017).

Current study was designed to decipher the impact of intronic variant rs1667627 on MTHFD2 expression in Pakistani population. Moreover, the correlation between MTHFD2 expression and bladder cancer stages was also determined. The MTHFD2 protein was further screened against a drug library from MCULE and FDA approved drugs to identify potential interacting drug using molecular docking approach.

Methodology

Collection of tissue sample and Tissue Treatment

In current study, the expression of genes related to the mitochondrial folate pathway such as MTHFD2 and SNP rs1667627 were investigated in the growth of bladder tumor. A 34 paired samples of bladder cancer patients were collected, 28 (82.3%) were of males, only 6 (17.6%) were of females. Mean age of patients included in this were 43.5±16.5 years (age range: 17- 70 years). Current study was conducted with a prior approval from the hospital and Department Ethical Review Board and all the samples were collected with a prior written consent of each patient. A sample of tumor size of approximately 5mm was collected. The surrounding healthy tissue separated by about 2 cm was collected from the same patient as a control. All the samples were immediately transferred to RNA later and were stored in a refrigerator at 2-8°C.

Sample preparation and RNA/DNA Isolation

RNA later was removed by shifting tissue to 1 ml of chilled PBS DEPC and washed at 4000 rpm for 5 min at 4°C. RNA extraction was performed using the previously reported Trizol reagent method (Chomczynski 1993). In phase separation during RNA extraction, the layer containing DNA was treated to isolate DNA by adding 200µl of 70% ethanol and after incubation of 4 minutes at room temperature, the centrifuged for 8 minutes at 13000 rpm. Supernatant was removed and the DNA pellet was store in isopropanol at -20°C.

The RNA quantification was performed by Nanodrop tool at A260/280. To assure the quality of extracted RNA and DNA, the RNA samples were loaded to 1.2% denaturing Agarose gel while DNA was quantified at 1% agarose gel and the band intensity was visualized under Ultra Violet light.

Primer Designing

RNA expression analysis was performed using exon spanning primers for targeted genes. The primers were designed by extracting cDNA sequence from Ensemble Genome Browser (<https://asia.ensembl.org/index.html>). dbSNP (<https://www.ncbi.nlm.nih.gov/snp/>) was used for extracting the DNA sequence flanking the SNP rs1667627. Primers were designed using primer Quest software (<https://sg.idtdna.com/pages/tools/primerquest>). The designed primers were validated using and UCSC in-silico PCR (<https://genome.ucsc.edu/cgi-bin/hgPcr>) and Primer Blast software (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The details of primers are shown in Supplementary Table I.

Quantitative Real time PCR

cDNA was synthesized using Revert-Aid first-strand cDNA synthesis kit (Cat no. K1622, Thermo Scientific, USA). The qPCR was performed using SYBR Green Master Mix (Maxima SYBR Green/ROX qPCR Master Mix (2X) Cat no. K0221, Thermo Scientific, USA) on real time PCR machine (Applied Biosystems, Step One qPCR system). *TUBB1* house-keeping gene was used in order to normalize the gene expression in both control and tumor samples. The relative fold change was analyzed by using $2^{-\Delta\Delta C_T}$ method as documented previously. The data was normalized with the endogenous control (*TUBB1*) and average fold increase was determined by calculating the relative expressions of each tumor sample. The normality of the data was tested using Shapiro-Wilk test and the outliers were removed using the Grubbs test at 0.05 level. The significance level of $P < 0.05$ calculated through T-test.

SnP Analysis

SNP analysis of rs1667627 was performed by amplifying the targeted region of DNA using specific primers (Supplementary Figure S2). The optimum conditions were; initial denaturation at 95°C for 5 min followed by a 34 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 35 sec and extension at 72°C for 30 sec. A final incubation of 72°C for 10 mins was followed. The amplification was confirmed by using 1.5% agarose gel and visualized under Ultra Violet. All the amplified products were sequenced from McLab, USA (<https://www.mclab.com/>) for SNP analysis.

In-silico drug screening against MTHFD2

Database preparation

High throughput screening library was prepared by combining FDA approved library from ZINC (<https://zinc.docking.org/>) database comprising of 1577 drug like molecules and 1483 molecules from MCULE Ro5 database (<https://mcule.com>). The unwanted molecules with improper conformation were removed and charges were assigned to the ligand molecules by using Autodock Vina.

Protein preparation

The protein was prepared for docking co-crystallized water molecules, non-standard residues, non-polar hydrogens were deleted. Hydrogens and Gasteiger charges were merged. Energy minimization and geometry optimization was performed using DockPrep built-in tool in UCSF chimera.10.1 (Resource for Biocomputing, Visualization, and Informatics, University of California San Francisco, San Francisco, CA, USA) (Pettersen, Goddard et al. 2004). The

energy was minimized for the 1000 steepest descent steps at a root means square gradient of 0.02, an update interval of 10, and with an Amber ff12SB force field using UCSF Chimera 10.1. Hydrogens were added in order to generate protonation states at physiological pH using energy minimization tool at UCSF Chimera (Pettersen, Goddard et al. 2004).

Grid Generation And Molecular Docking Simulation

The prepared crystal structure of MTHFD2 was used as a receptor and grid box around the active site was generated. The center of the grid was defined at X=98.59, Y=15.0818, Z=-2.6543

with 25.3382 Å length in X, 16.4734 Å length in Y and 21.1438 Å length in Z dimension. During docking simulations top 9 poses of the compounds were subjected to minimization and best poses were selected based on the lowest binding affinity score calculated in kcal/mol.

Results

In-silico analysis of MTHFD2 expression

Expression analysis of MTHFD2 was carried out on TCGA dataset comprising of 399 tumor samples and 19 normal samples. Significant up-regulation of MTHFD2 was observed in non-papillary bladder carcinoma as compared to normal. However, no distinct difference was observed between papillary carcinoma and normal (Figure 1A). Disease free patient survival analysis of MTHFD2 showed less survival probability of high expression group (shown in red) whereas the low expression group (shown in blue) (Figure 1B) exhibited higher survival probability indicating the oncogenic potential of MTHFD2.

Expression analysis of MTHFD2 in different grades of bladder cancer

The *in-silico* analysis of MTHFD2 was validated in different grades of bladder cancer. The bladder cancer was classified into papillary, low grade and high-grade bladder cancer. Relative expression analysis of MTHFD2 was low in high grade T1 stage as compared to controls (Figure 2A, S1). However, a significant 8-fold increase in mRNA levels of MTHFD2 was observed at the T2 stage of high grade bladder cancer. (Figure 2A). However, in this study significant increase in MTHFD2 expression was observed in T1 and T2 stage low bladder cancer around 21 and 52 folds as compared to normal (Figure 2B, S1). This indicates that MTHFD2 is involved prominently in low grade cancer progression. Although the TCGA data based analysis of MTHFD2 did not show significant upregulation in papillary bladder cancer. However, a significant increase in MTHFD2 expression was observed at T1 and T2 stage papillary bladder cancer around 5 and 34 folds change at RNA level as compared to controls (Figure 2C, S1) indicating that MTHFD2 is more prominently associated with less invasive bladder cancer subtypes.

SNP Analysis

The high expression of MTHFD2 was further correlated with an intronic variant SNP rs1667627. High bladder cancer susceptibility is associated with this intronic variant in Western population (Andrew, Gui et al. 2009). In normal samples this intronic variant displayed a heterozygous pattern A/G whereas in the tumor sample homozygous A/A genotype was observed. This variation from G to A might increase the susceptibility to bladder cancer risk (Figure 3, S2).

Structure based virtual screening

A virtual screening library was compiled by combining ZINC FDA approved drugs library and M-CULE Ro5 library in order to find new drug based inhibitors against MTHFD2 (PDB ID 5TC4) using AutoDock VINA implicated in the PyRx tool. 9 different conformations for each ligand were generated. These ligands based interaction were then classified based on binding affinity (kcal/mol). The top 5 ranked compounds displaying the free energy of binding in the range -14.3 to -9.3kcal/mol are presented in Table I. All these selected ligands had lower binding free energy than LY345899 (Gustafsson, Jemth et al. 2017). The docking poses of the candidates are shown in Figure 4 along with the docking pose of LY345899. The interaction analysis of the top scoring ligands showed lower binding energy and four hydrogen bonds with MCULE-8027924848.

Prediction of ADMET properties

Molecular descriptors are the critical factors to determine the pharmacokinetic properties and toxicity of the candidate compounds. In-silico ADMET properties prediction aid to identify the potential therapeutic compounds. The BBB, aqueous solubility, CYP450 inhibition, Lipinski rule violation, PAINS evaluation for toxicity determination were used for screening for potential

ADMET properties predicted. Table II & III showed the ADMET profile of the selected ligands. analysis of the top scoring ligands showed lower binding energy and four hydrogen bonds with MCULE-8027924848 showed no violation of Lipinski rules with high GI absorption and no liver toxicity indicating its therapeutic potential against MTHFD2.

Discussion

Urinary bladder cancer is a multifactorial disorder owing to diverse genetic and environmental etiologies. It is regarded as one of the most common urological malignancy worldwide exhibiting male predominance (Ali, Bangash et al. 2017). The folate metabolism pathway provides the 1C units in the form of formyl groups for *de-novo* purine and thymidine synthesis, that are critical for cell proliferation. Apart from this, MTHFD2 maintains the redox homeostasis under oxidative stress condition during hypoxia or extracellular matrix detachment (Ju, Lu et al. 2019). TCGA based data analysis revealed significant upregulation of MTHFD2 in non-papillary bladder cancer (Figure 1A). The high expression group also displayed low survival probability TCGA dataset (Figure 1B). Tissue based expression analysis from indigenous samples showed progressive strong increase in MTHFD2 expression at RNA level both in low grade and papillary bladder cancer as compared to control. However, the strongest fold change difference was observed in low grade bladder cancer around 21 and 52 folds as compared to 5 and 34 folds increase in papillary bladder cancer indicating MTHFD2 expression correlation with low grade bladder cancer progression (Figure 2B & C). However, a significant 8-fold increase in MTHFD2 expression was observed at the T2 stage of high grade bladder cancer (Figure 2A). Homozygous variant of SNP rs1667627 in MTHFD2 gene has been associated with bladder cancer risk (Andrew, Gui et al. 2009). The increased expression of MTHFD2 was observed in papillary bladder cancer apart from the non-papillary cancer in the current study which further intrigued to see the impact of this SNP in Pakistani population. A homozygous A/A genotype was observed in this intronic SNP variant in tumor samples as compared to controls exhibiting the heterozygous A/G genotype. Furthermore, structure based virtual ligand library screening revealed a plausible inhibitor (MCULE-8027924848) of MTHFD2. This ligand displayed lower binding free energy -14.3 kcal/mol as compared to the previously documented LY3458993 (-9.3 kcal/mol (Table I)) (Gustafsson, Jemth et al. 2017). The compound displayed low toxicity at cellular and tissue level indicating its potential as a novel MTHFD2 inhibitor (Table II & III).

However, future pharmacological studies are required to determine its optimal inhibitory concentration against bladder cancer cells and mode of inhibition.

Conclusion

In this study, the expression of genes related to the mitochondrial folate pathway such as MTHFD2 and SNPs rs1667627 were investigated in the growth of bladder tumor. MTHFD2 is highly express in low grade bladder tumor stages. Homozygous genotype in SNPs rs1667627 variant was associated with tumor tissues. The virtual ligand based screening against MTHFD2 lead to a potential inhibitor. However, future studies are required to determine its potential mode of action.

Declarations

COMPLIANCE with ETHICAL STANDARDS

FUNDING INFORMATION

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the ethical review board of COMSATS University Islamabad (approval no. CUI/Bio/ERB/18/76). Written signed consent was obtained from the patients involved.

PATIENT CONSENT FOR PUBLICATION

Written signed consent was obtained from the patients involved.

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References

1. Ali SHB et al (2017) "Identification of novel potential genetic predictors of urothelial bladder carcinoma susceptibility in Pakistani population. " *Familial cancer* 16(4):577–594
2. Andrew AS et al (2009) "Bladder cancer SNP panel predicts susceptibility and survival". *Hum Genet* 125(5–6):527–539
3. Chomczynski P (1993) A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* 15(3):532–534
4. Ducker GS, Rabinowitz JD (2017) One-carbon metabolism in health and disease. *Cell Metabol* 25(1):27–42
5. Gustafsson R et al (2017) "Crystal structure of the emerging cancer target MTHFD2 in complex with a substrate-based inhibitor. " *Cancer Research* 77(4):937–948
6. Ju H-Q et al (2019) Modulation of redox homeostasis by inhibition of MTHFD2 in colorectal cancer: mechanisms and therapeutic implications. *JNCI: Journal of the National Cancer Institute* 111(6):584–596
7. Liu F et al (2014) "Increased MTHFD2 expression is associated with poor prognosis in breast cancer". *Tumor Biology* 35(9):8685–8690
8. Oliveira AI et al (2012) Moving forward in bladder cancer detection and diagnosis: the role of epigenetic biomarkers. *Expert Rev Mol Diagn* 12(8):871–878
9. Pettersen EF et al (2004) UCSF Chimera—a visualization system for exploratory research and analysis. *J Comput Chem* 25(13):1605–1612
10. Siegel R et al (2012) "Cancer statistics for hispanics/latinos, 2012." *CA: a cancer. journal for clinicians* 62(5):283–298
11. Siegel RL et al (2016) "Cancer statistics, 2016." *CA: a. cancer journal for clinicians* 66(1):7–30
12. Tedeschi PM et al (2015) "Mitochondrial methylenetetrahydrofolate dehydrogenase (MTHFD2) overexpression is associated with tumor cell proliferation and is a novel target for drug development". *Mol Cancer Res* 13(10):1361–1366
13. Tian W et al (2018) CASTp 3.0: computed atlas of surface topography of proteins. *Nucleic Acids Res* 46(W1):W363–W367
14. Tunio MA et al (2011) "Outcome of trimodality protocol for invasive bladder cancer patients at Karachi. Pakistan " *JPM-A:Journal of the Pakistan Medical Association* 61(9):874
15. Yang M, Vousden KH (2016) "Serine and one-carbon metabolism in cancer". *Nat Rev Cancer* 16(10):650–662

Tables

Table I: Top five ligands with binding affinity against MTHFD2 (5TC4)

Sr.No	Ligand	Binding Affinity (Kcal/mol)
1	MCULE-8109969891-0	-14.3
2	MCULE-8027924848	-14.1
3	MCULE-2077919807-2	-13.9
4	MCULE-3376329397-0	-13.2
5	ZINC000253632968	-10.2
	LY3458993	-9.3

Table II: Structural characteristics of Ligands

Sr.No.	Ligands	Canonical SMILES	Formula	MW	#Heavy atoms	#Aromatic heavy atoms
1	MCULE-8109969891-0	<chem>OC(=O)CCC(C(=O)O)NC(=O)c1ccc(cc1)N1CC2N(C1=O)c1c(NC2)[nH]c(nc1=O)N</chem>	C20H21N7O7	471.42	34	12
2	MCULE-80279848	<chem>COc1ccc(cc1)CC(=O)NCCN1CCN(CC1)C(=O)c1cn(nc1OC)C</chem>	C21H29N5O4	415.49	30	11
3	MCULE-2077919807-2	<chem>O=CN1C(CNc2ccc(cc2)C(=O)NC(C(=O)O)CCC(=O)O)CNc2c1c(=O)nc([nH]2)N</chem>	C20H23N7O7	473.44	34	12
4	MCULE-3376329397-0	<chem>CCOc1cccc2c1Oc1c(C2)c(=O)n(n1CC(=O)Nc1ccc2c(c1)OCCO2)c1ccc(cc1)CC</chem>	C30H29N3O6	527.57	39	23

Table III: ADMET profile of potential five multi-targeted virtual hits

Sr.No.	Ligands	ESOL Class	Membrane transporter		Metabolism					Lipinski #violations	Liver toxicity			GI absorption	PAINS alert
					Cyp inhibitors for						DILI	Cyto-toxicity			
			BBB perm-eant	Pgp subst-rate	1A2	2C19	2C9	2D6	3A4						
1	MCULE-8109969891-0	Very soluble	No	No	No	No	No	No	No	2	No	Yes	Low	0	
2	MCULE-80279848	Soluble	No	Yes	No	No	No	No	Yes	0	No	No	High	0	
3	MCULE-2077919807-2	Very soluble	No	No	No	No	No	No	No	2	No	Yes	Low	0	
4	MCULE-3376329397-0	Poorly soluble	No	Yes	No	Yes	Yes	Yes	Yes	1	Yes	No	High	0	
5	ZINC253632968	Poorly soluble	No	Yes	No	No	No	No	Yes	2	Yes	No	Low	0	

Figures

Figure 1

Figure 1

(A). Relative expression analysis of MTHFD2 in bladder cancer molecular subtypes using GEPIA analysis (<http://gepia.cancer-pku.cn/>) (Z. Tang et al., 2017) software based on the TCGA and Genotype –Tissue Expression (GTEx) database using $|\log FC| \geq 1$ and $P \leq 0.05$ as the cut-off criteria. The Y-axis represent s the $\log_2 (TPM+1)$ expression values of the respective gene. MTHFD 2 showed significant increased expression in non-papillary subtype of bladder cancer. (B). The survival data indicates that in the bladder cancer patients showed significantly reduced disease free survival in MTHFD2 high expression group represented as red line as compared to the MTHFD2 low expression group shown in blue. The X –axis represents the number of months.

Figure 2

(A). RT-qPCR showing relatively high levels of MTHFD2 at T2 stage in high grade bladder cancer as compared to control and T1 stage. (B). RT-qPCR expression analysis at mRNA level showed relatively increased levels of MTHFD2 at T1 and T2 stage in low grade bladder cancer as compared to control samples. (C). Expression analysis at mRNA level showed increased levels of MTHFD2 at T1 and T2 stage in papillary bladder cancer as compared to control samples. However, the increase is more pronounced at the T2 stage as compared to the T1 stage.

Figure 3 SNPrs1667627

Figure 3 DNA sequencing analysis of the SNP rs166727 Electropherogram showed heterozygous A/G genotype in normal tissue as compared to tumor sample exhibiting the homozygous A/A genotype.

Figure 4 Surface representation of the best docking poses of the selected ligands and its comparison with the already documented ligand LY3458993

FIGURE 5

Figure 5

Ligplot+ based interaction analysis of the MTHFD2 and ligands docked complexes. Green dashed lines indicate hydrogen bonds with distance in Angstrom ($\text{\AA}$), spoked red arcs indicate hydrophobic contacts, atoms are shown in black for carbon, blue for nitrogen, red represents oxygen and yellow represents sulfur.

Supplementary Files

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