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Article

Cell Cycle Regulation and DNA Damage Response Networks in Diffuse-and Intestinal-Type Gastric Cancer.

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Simple Summary: Epithelial-mesenchymal transition (EMT) is an important hallmark in drug resistance and cancer malignancy in cancer stem cells (CSCs). In this study, gene expression in diffuse- and intestinal-type gastric cancer (GC) was investigated to reveal the precise mechanism of EMT. The pathways on cell cycle regulation and DNA damage response were found to be altered in diffuse- and intestinal-type GC. The findings of this study may provide broader insights into CSCs with new possibilities of the involvement of the cell cycle in EMT.

Abstract: Epithelial-mesenchymal transition (EMT) networks are essential in acquiring the drug resistance and cancer malignant features in cancer stem cells (CSCs). In this regard, gene expression profiles in diffuse- and intestinal-type gastric cancer (GC) have been analyzed to reveal the network pathways in EMT and CSCs, since the diffuse-type GC has much more mesenchymal features than intestinal-type GC that has the intestinal features. The study results revealed that the activation state of several canonical pathways related to cell cycle regulation was altered. The canonical pathway on Cell cycle: G_1/S checkpoint regulation was activated in diffuse-type GC, and canonical pathways on Cell cycle control of chromosomal replication and Cyclins and cell cycle regulation were activated in intestinal-type GC. Canonical pathway related to Role of BRCA1 in DNA damage response was activated in intestinal-type GC, where BRCA1, which is related to G_1/S phase transition was upregulated in intestinal-type GC. Several microRNAs (miRNAs), including mir-10, mir-17, mir-19, mir-194, mir-224, mir-25, mir-34, mir-451, and mir-605, were identified to have direct relationships of RNA-RNA interaction in Cell cycle: G_1/S checkpoint regulation pathway. Additionally, cell cycle regulation may be altered in EMT conditions. The alterations in activation states of the pathways related to cell cycle regulation in diffuse- and intestinal-type GC would indicate the significance of cell cycle regulation in EMT.

Keywords: BRCA1; cancer stem cell; cell cycle; epithelial-mesenchymal transition; DNA damage response; gastric cancer; molecular network

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1. Introduction

In cancer stem cells (CSCs) in general, epithelial-mesenchymal transition (EMT) networks play an important role in acquiring the drug resistance and cancer malignant feature [1]. Alteration in gene expression of molecular network pathways results in the phenotypic changes. Diffuse-type GC has much more mesenchymal characteristics, which is an important feature of EMT, compared to intestinal-type GC. Accordingly, gene expression profiles have been analyzed for diffuse- and intestinal-type gastric cancer (GC) to reveal the network pathways in EMT and CSCs. Our previous findings identified several molecular networks and the related microRNAs (miRNAs) in intestinal- and diffuse-type GC [2,3]. Again, few recent studies have revealed the regulation of non-coding RNAs, including miRNAs in drug resistance and EMT in cancer [4-6]. It has been also been featured that the knockdown of circularNOP10, a circular RNA promoting tumor metastasis and EMT, decreased the numbers of cells in the S phase and increased the number of cells in the G_2/M phase [6].

The gene expression signature revealed that the ratio of CDH2 (N-cadherin) to CDH1 (E-cadherin) distinguishes the diffuse- and intestinal-type GC [7]. The gene expression and pathway analysis of CTNNB1 (β-catenin) identified an important role of β-catenin signaling in the regulation of stem cell pluripotency and cancer signaling [8]. Although cell proliferation and cell cycle regulation are essential for identifying potential therapeutic targets in cancer, the precise mechanism of cell cycle regulation and EMT has not been fully revealed. This article focuses on the roles of cell cycle regulation pathways in diffuse- and intestinal-type GC as cell cycle regulation may play a critical role in intestinal- and diffuse-type GC. Consequently, the mechanism of cancer drug resistance is highlighted through the involvement of the cell cycle in EMT and CSCs, which might reveal future therapeutic potentials.

2. Materials and Methods

2.1. Data Analysis of Diffuse- and Intestinal-Type GC

The RefSeq data of diffuse- and intestinal-type GC are publicly available in The Cancer Genome Atlas (TCGA) of The cBioPortal for Cancer Genomics database [9-11] in National Cancer Institute (NCI) Genomic Data Commons (GDC) Data Portal [12]. From the publicly available data of stomach adenocarcinoma in TCGA [10], intestinal- and diffuse-type GC data, which are noted as chromosomal instability (CIN) (n=223) and genomically stable (GS) (n=50), respectively, in TCGA Research Network publication, were compared [11].

2.2. Network Analysis

Data of intestinal- and diffuse-type GC in TCGA cBioPortal Cancer Genomics were uploaded and analyzed through the use of Ingenuity Pathway Analysis (IPA) (QIAGEN Inc., Hilden, Germany) [13].

2.3. Data Visualization

The results of gene expression data of RefSeq and network analysis were visualized by Tableau software (https://www.tableau.com).

2.4. Statistical Analysis

The RefSeq data were analyzed by Student's t-test. Z-score in intestinal- and diffuse-type GC samples were compared, and the difference was considered to be significant in p value < 0.00001 [2].

3. Results

3.1. Canonical Pathways in Diffuse- and Intestinal-Type GC

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Canonical pathways altered in diffuse- and intestinal-type GC are shown in Figure 1 and Table 1. The 2815 IDs which are significantly different in diffuse- and intestinal-type GC were analyzed in network analysis, which identified 47 canonical pathways with absolute z-score > 1 in the diffuse- and intestinal-type GC (Table 1). These canonical pathways included Cell cycle control of chromosomal replication, Cell cycle: G_1/S checkpoint regulation, Cyclins and cell cycle regulation, Role of BRCA1 in DNA damage response, and Cell cycle: G_2/M DNA damage checkpoint.

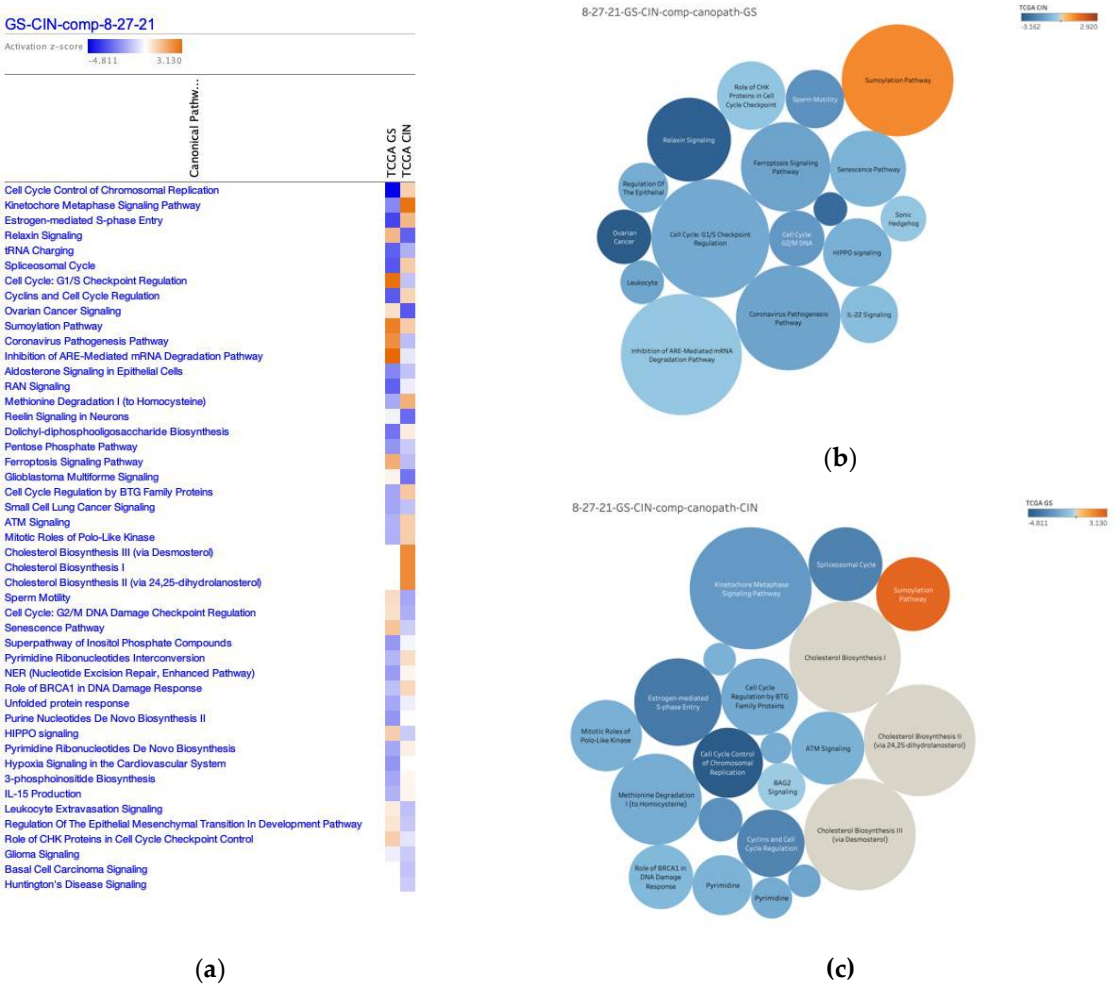

Figure 1. Canonical pathways altered in diffuse- and intestinal-type GC. (**a**) Canonical pathways with absolute z-score > 1 in the diffuse- and intestinal-type GC are shown (TCGA GS; diffuse-type GC, TCGA CIN; intestinal-type GC); (**b**) Size shows the activation score in diffuse-type GC. Color indicates the activation score in intestinal-type GC.; (**c**) Size indicates the activation z-score in intestinal-type GC. Color indicates the activation z-score in intestinal-type GC.

Table 1. Canonical pathways altered in diffuse- and intestinal-type GC. The pathways are sorted by the order of the activation z-score.

Canonical Pathways	Diffuse-type GC	Intestinal-type GC
Cell Cycle Control of Chromosomal Replication	-4.811	0.962
Kinetochore Metaphase Signaling Pathway	-2.271	2.92
Estrogen-mediated S-phase Entry	-3.5	1.5
Relaxin Signaling	1.5	-3
tRNA Charging	-3	-1.5
Spliceosomal Cycle	-3.207	1.069
Cell Cycle: G_1/S Checkpoint Regulation	2.982	-1.147

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Cyclins and Cell Cycle Regulation	-3.13	0.894
Ovarian Cancer Signaling	0.632	-3.162
Sumoylation Pathway	2.673	1.069
Coronavirus Pathogenesis Pathway	2.335	-1.257
Inhibition of ARE-Mediated mRNA Degradation Pathway	3.13	-0.447
Aldosterone Signaling in Epithelial Cells	-2.309	-1.155
RAN Signaling	-3	-0.333
Methionine Degradation I (to Homocysteine)	-1.633	1.633
Reelin Signaling in Neurons	-0.218	-2.837
Dolichyl-diphosphooligosaccharide Biosynthesis	-2.646	0.378
Pentose Phosphate Pathway	-2	-1
Ferroptosis Signaling Pathway	1.706	-1.279
Glioblastoma Multiforme Signaling	0.243	-2.668
Cell Cycle Regulation by BTG Family Proteins	-1.732	1.155
Small Cell Lung Cancer Signaling	-1.732	-1.155
ATM Signaling	-1.46	1.043
Mitotic Roles of Polo-Like Kinase	-1.5	1
Cholesterol Biosynthesis III (via Desmosterol)	0	2.449
Cholesterol Biosynthesis I	0	2.449
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	0	2.449
Sperm Motility	0.728	-1.698
Cell Cycle: G_2/M DNA Damage Checkpoint Regulation	0.655	-1.528
Senescence Pathway	1.234	-0.926
Superpathway of Inositol Phosphate Compounds	-1.976	-0.18
Pyrimidine Ribonucleotides Interconversion	-1.414	0.707
NER (Nucleotide Excision Repair, Enhanced Pathway)	-1.877	0.209
Role of BRCA1 in DNA Damage Response	-1.225	0.816
Unfolded protein response	-1.667	-0.333
Purine Nucleotides De Novo Biosynthesis II	-2	0
HIPPO signaling	1	-1
Pyrimidine Ribonucleotides De Novo Biosynthesis	-1.667	0.333
Hypoxia Signaling in the Cardiovascular System	-2	0
3-phosphoinositide Biosynthesis	-1.671	0.186
IL-15 Production	-1.46	0.209
Leukocyte Extravasation Signaling	0.408	-1.225
Regulation Of The Epithelial Mesenchymal Transition In Development Pathway	0.535	-1.069
Role of CHK Proteins in Cell Cycle Checkpoint Control	1	-0.5
Glioma Signaling	-0.333	-1
Basal Cell Carcinoma Signaling	0	-1.155
Huntington's Disease Signaling	0	-1

3.2. Cell Cycle-Related Canonical Pathways in Diffuse- and Intestinal-Type GC

3.2.1. Cell cycle control of chromosomal replication was activated in intestinal-type GC

Molecule activity predictor in IPA predicted the activation of Cell Cycle Control of Chromosomal Replication pathway in intestinal-type GC (Figure 2). Analysis on direct relationships of RNA-RNA interactions: microRNA targeting in Cell Cycle Control of Chromosomal Replication pathway revealed the relationships between miRNAs and the targeted molecules (Table 2).

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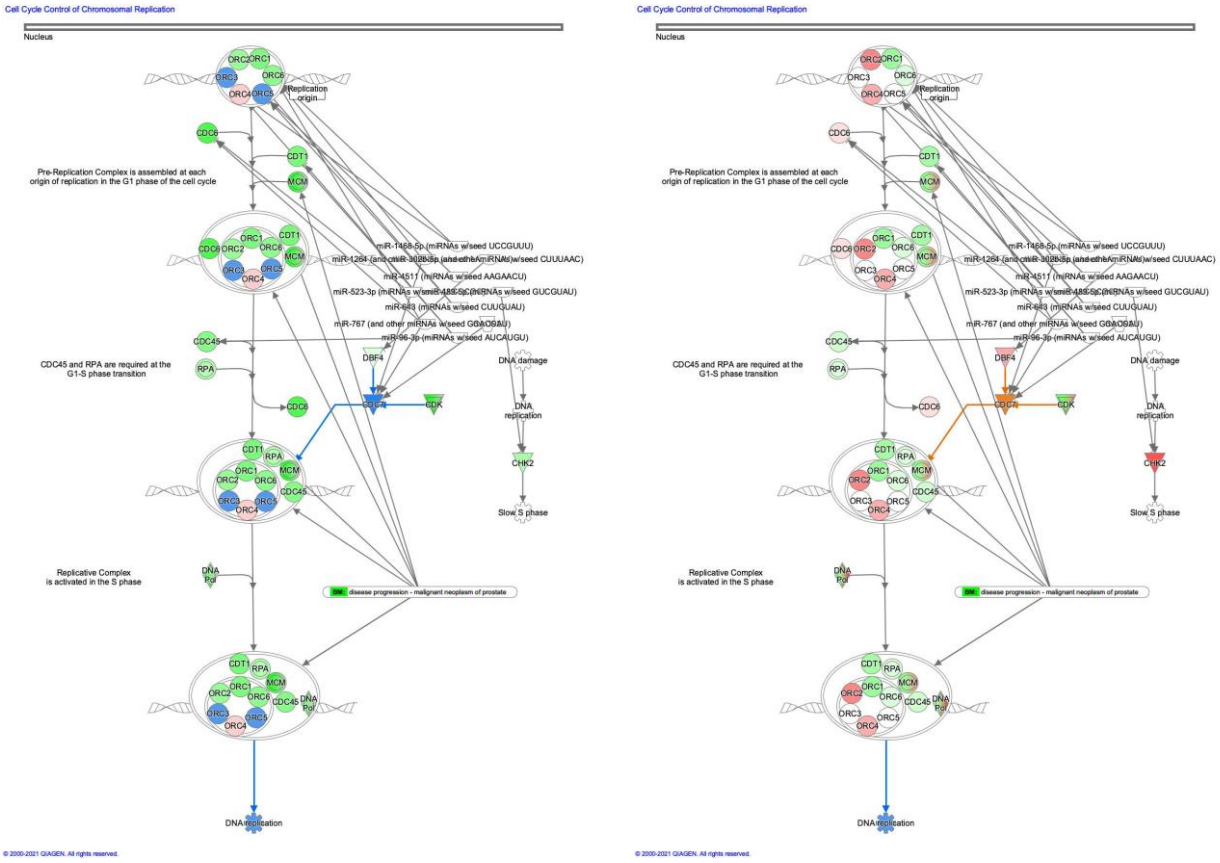

(**a**) (**b**)

Figure 2. Cell cycle control of chromosomal replication was activated in intestinal-type GC. (**a**) Gene expression and pathway activity prediction in diffuse-type GC are shown; (**b**) Gene expression and pathway activity prediction in intestinal-type GC are shown. The genes of which expression was altered in diffuse- and intestinal-type GC are shown in pink (up-regulated) or green (down-regulated). Predicted activation or inhibition is shown in orange or blue, respectively.

Table 2. Direct relationships of miRNAs and targeted molecules in Cell cycle control of chromosomal replication.

From Molecule(s)	To Molecule(s)
miR-1264 (and other miRNAs w/seed AAGUCUU)	CDC7
	ORC6
miR-1468-5p (miRNAs w/seed UCCGUUU)	ORC4
	ORC6
mir-192	CDC7
miR-302b-5p (and other miRNAs w/seed CUUUAAC)	DBF4
miR-4511 (miRNAs w/seed AAGAACU)	ORC5
miR-489-5p (miRNAs w/seed GUCGUAU)	CHK2
miR-523-3p (miRNAs w/seed AACGCGC)	CDC6
miR-643 (miRNAs w/seed CUUGUAU)	ORC2
	ORC5
miR-767 (and other miRNAs w/seed GCACCAU)	CDC6
	CDC7
	ORC6
miR-96-3p (miRNAs w/seed AUCAUGU)	CDC45
	CDT1
	ORC4

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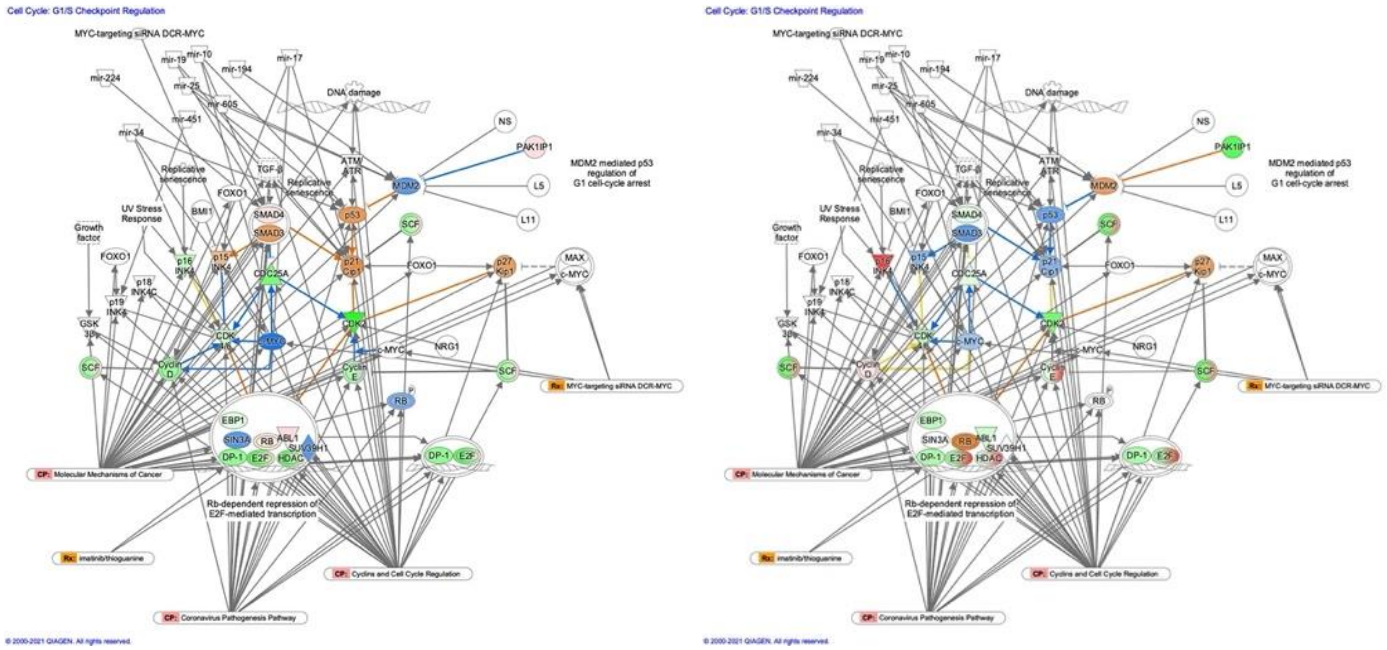
3.2.2. Cell cycle: G1/S checkpoint regulation pathway was activated in diffuse-type GC

Molecule activity predictor in IPA predicted the activation of Cell cycle: G1/S checkpoint regulation pathway in diffuse-type GC (Figure 3). In Cell cycle: G1/S checkpoint regulation pathway, DNA damage induces p53, which is expected to be activated in diffuse-type GC. Analysis on direct relationships of RNA-RNA interaction in Cell cycle: G1/S checkpoint regulation pathway revealed the non-coding RNAs including 9 miRNAs and a biologic drug (MYC-targeting siRNA DCR-MYC) (Table 3).



(**a**) (**b**)

Figure 3. Cell cycle: G1/S checkpoint regulation pathway was activated in diffuse-type GC. (**a**) Gene expression and pathway activity prediction in diffuse-type GC are shown; (**b**) Gene expression and pathway activity prediction in intestinal-type GC are shown. The genes of which expression was altered in diffuse- and intestinal-type GC are shown in pink (up-regulated) or green (down-regulated). Predicted activation or inhibition is shown in orange or blue, respectively.

Table 3. Non-coding RNAs which have direct relationships in Cell cycle: G1/S checkpoint regulation pathway.

Symbol	Entrez Gene Name	Location	Family
mir-10	microRNA 99a	Cytoplasm	microRNA
mir-17	microRNA 17		
mir-19	microRNA 19a		
mir-194	microRNA 194-1		
mir-224	microRNA 224		
mir-25	microRNA 25		
mir-34	microRNA 34a		
mir-451	microRNA 451a		
mir-605	microRNA 605		
MYC-targeting siRNA DCR-MYC		Other	biologic drug

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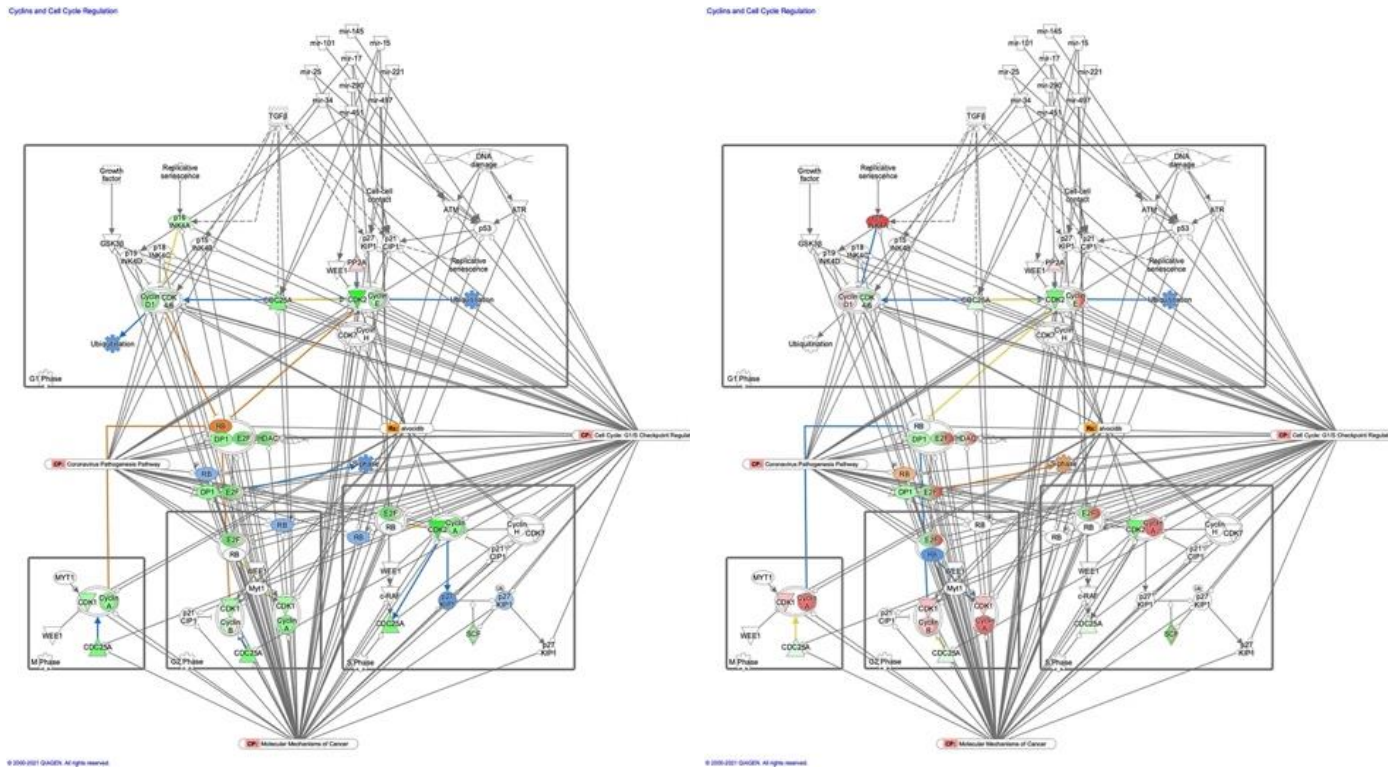
3.2.3. Cyclins and cell cycle regulation pathway was activated in intestinal-type GC

Molecule activity predictor in IPA predicted the activation of Cyclins and cell cycle regulation pathway in intestinal-type GC (Figure 4). Analysis on direct relationships of RNA-RNA interactions: microRNA targeting in Cyclins and cell cycle regulation pathway revealed the relationships between miRNAs and the targeted molecules (Table 4).



(**a**) (**b**)

Figure 4. Cyclins and cell cycle regulation was activated in intestinal-type GC. (**a**) Gene expression and pathway activity prediction in diffuse-type GC are shown; (**b**) Gene expression and pathway activity prediction in intestinal-type GC are shown. The genes of which expression was altered in diffuse- and intestinal-type GC are shown in pink (up-regulated) or green (down-regulated). Predicted activation or inhibition is shown in orange or blue, respectively.

Table 4. Direct relationships of miRNAs and targeted molecules in Cyclins and cell cycle regulation.

From Molecule(s)	To Molecule(s)
mir-10	ATM
mir-145	p53
mir-15	CDC25A
	WEE1
	c-RAF
mir-17	ATM
	CyclinD1
	RB
	p21CIP1
mir-221	p27KIP1
mir-25	p21CIP1
	p53
mir-290	CDK2
mir-34	CDK4/6
	p53
mir-451	p19INK4D

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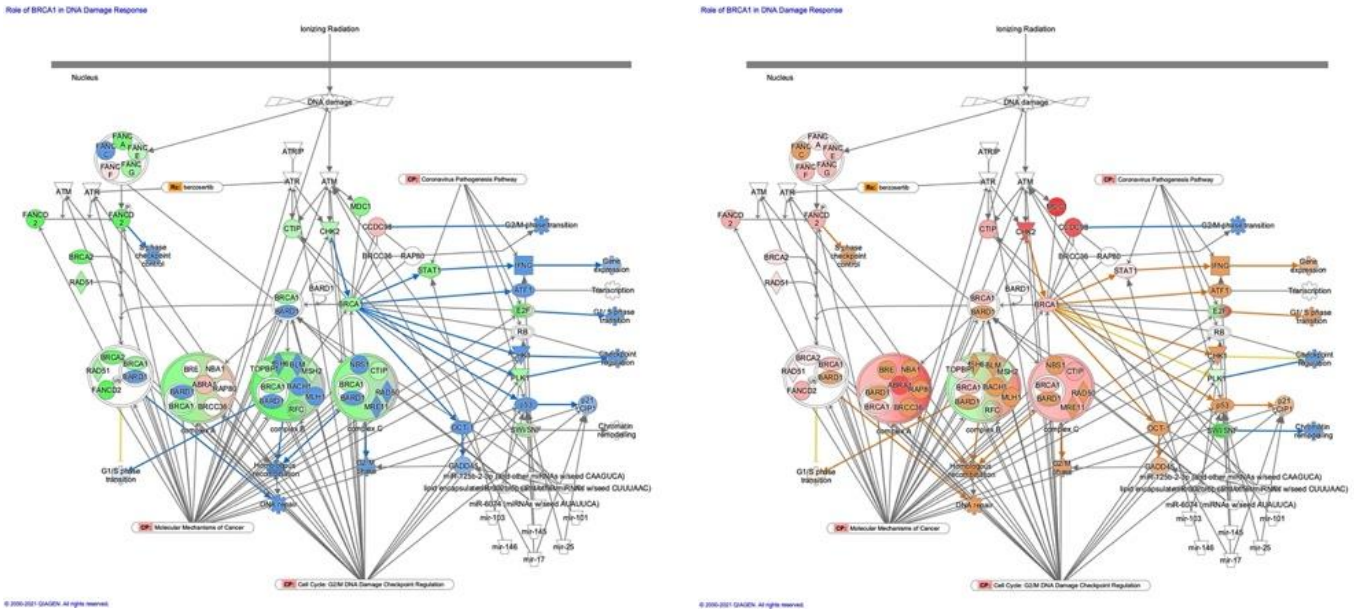
mir-497	CDC25A
	c-RAF

3.2.4. Role of BRCA1 in DNA damage response pathway was activated in intestinal-type GC

Molecule activity predictor in IPA predicted the activation of Role of BRCA1 in DNA damage response pathway in intestinal-type GC (Figure 5). Role of BRCA1 in DNA damage response pathway was identified as the most significant canonical pathway with p value of 6.6×10^{-12}. Gene expression of BRCA1 which is associated with G_1/S transition has increased in intestinal-type GC. BRCA1 codes a 190kD nuclear phosphorylation protein that maintains genomic stability and functions as a tumor suppressor. It is interesting that p53 and c21CIP1 are activate in intestinal-type GC in the Role of BRCA1 in DNA damage response pathway. BRCA1 may be involved in the activation of p53. Analysis on direct relationships of RNA-RNA interactions: microRNA targeting in Role of BRCA1 in DNA damage response pathway revealed the relationships between miRNAs and the targeted molecules (Figure 5, Table 5).



(**a**) (**b**)

Figure 5. Role of BRCA1 in DNA damage response was activated in intestinal-type GC. (**a**) Gene expression and pathway activity prediction in diffuse-type GC are shown; (**b**) Gene expression and pathway activity prediction in intestinal-type GC are shown. The genes of which expression was altered in diffuse- and intestinal-type GC are shown in pink (up-regulated) or green (down-regulated). Predicted activation or inhibition is shown in orange or blue, respectively.

Table 5. Direct relationships of miRNAs and targeted molecules in Role of BRCA1 in DNA damage response.

From Molecule(s)	To Molecule(s)
miR-125b-2-3p (and other miRNAs w/seed CAAGUCA)	p53
miR-302b-5p (and other miRNAs w/seed CUUUAAC)	BARD1
	CTIP
	GADD45
miR-6074 (miRNAs w/seed AUAUUCA)	FANCF
	IFNG
	NBS1
mir-101	ATM
mir-103	p53

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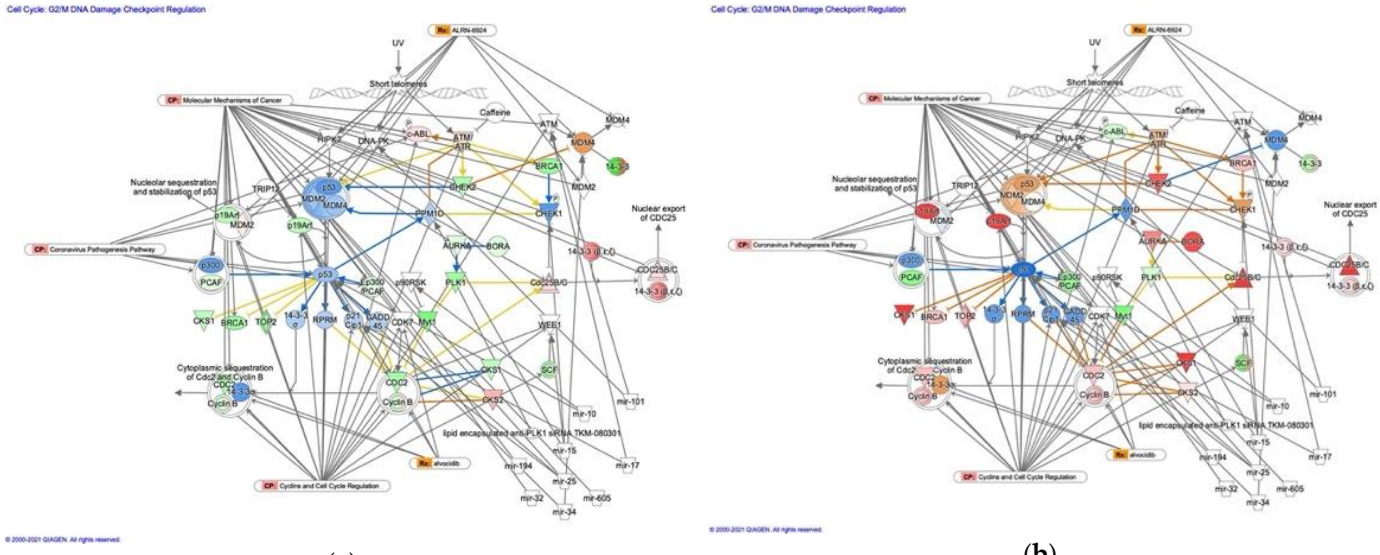
mir-145	p53
mir-146	STAT1
mir-17	ATM
	RB
	p21CIP1
mir-25	p21CIP1
	p53

3.2.5. Cell cycle: G2/M DNA damage checkpoint regulation pathway in diffuse- and intestinal- type GC

Cell cycle: G2/M DNA damage checkpoint regulation pathway was identified as related canonical pathway in diffuse- and intestinal- type GC (Figure 6). Analysis on direct relationships of RNA-RNA interaction in Cell cycle: G2/M DNA damage checkpoint regulation pathway revealed the non-coding RNAs including 9 miRNAs and a biologic drug (lipid encapsulated anti-PLK1 siRNA TKM-080301) (Table 6).



(**a**) (**b**)

Figure 6. Cell cycle: G2/M DNA damage checkpoint regulation pathway in diffuse- and intestinal- type GC. (**a**) Gene expression and pathway activity prediction in diffuse-type GC are shown; (**b**) Gene expression and pathway activity prediction in intestinal-type GC are shown. The genes of which expression was altered in diffuse- and intestinal-type GC are shown in pink (up-regulated) or green (down-regulated). Predicted activation or inhibition is shown in orange or blue, respectively.

Table 6. Direct relationships of miRNAs and targeted molecules in Cell cycle: G2/M DNA damage checkpoint regulation pathway.

Symbol	Entrez Gene Name	Location	Family
lipid encapsulated anti-PLK1 siRNA TKM-080301		Other	biologic drug
mir-10	microRNA 99a	Cytoplasm	microRNA
mir-101	microRNA 101-1		
mir-15	microRNA 15a		
mir-17	microRNA 17		
mir-194	microRNA 194-1		
mir-25	microRNA 25		
mir-32	microRNA 32		
mir-34	microRNA 34a		
mir-605	microRNA 605		

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4. Discussion

From the results of network analysis in diffuse- and intestinal-type GC, several canonical pathways related to cell cycle regulation including "Cell cycle control of chromosomal replication", "Cell cycle: G_1/S checkpoint regulation", "Cyclins and cell cycle regulation", and "Cell cycle: G_2/M DNA damage checkpoint regulation" have been identified to alter. A previous study showed that G_1/S arrest induces EMT by ribosome biogenesis [14]. This finding of G_1/S arrest in EMT may be associated with the finding in the current study in terms of activating the "Cell cycle: G_1/S checkpoint regulation" pathway in diffuse-type GC. SMAD4, which was activated in diffuse-type GC, is involved in EMT [15]. The silencing of SMAD4 reversed EMT in hepatocytes [16]. The several miRNAs have been found to have direct relationships in pathways related to cell cycle regulation. In "Cell cycle: G_1/S checkpoint regulation", mir-10 (microRNA 99a), mir-17 (microRNA 17), mir-19 (microRNA 19a), mir-194 (microRNA 194-1), mir-224 (microRNA 224), mir-25 (microRNA 25), mir-34 (microRNA 34a), mir-451 (microRNA 451a), mir-605 (microRNA 605), and MYC-targeting siRNA DCR-MYC were identified as molecules having the direct relationships (RNA-RNA interactions). It has been previously revealed that miR-17/20 cluster, which is transcriptionally regulated by MYC, E2F, and cyclin D1, and miR-34, is a direct transcriptional target of p53 and regulates cell cycle [17]. The cell cycle regulation of miRNAs can be involved in cancer. Small nucleolar RNA host gene 7 (SNHG7), an oncogenic long non-coding RNA, promotes cell migration via miR-34a-Snail-EMT axis in gastric cancer [18]. The involvement of miR-34 in EMT and GC is consistent with the results showing the direct relationship between miR-34 and "Cell cycle: G_1/S checkpoint regulation" pathway, which is activated in diffuse-type GC. Another study suggested that miRNA-33a inhibited EMT and metastasis of GC via the Snail/Slug pathway, which may be related to the miRNA regulation in EMT in intestinal-type GC [19]. MYC-targeting siRNA, a biologic drug targeting MYC, has been identified to have direct relationship in "Cell cycle: G_1/S checkpoint regulation". It has been shown before that the silencing of Aurora kinase B, which is up-regulated in GC, decreased the expression of MYC, arrested the cell cycle in G_2/M phase, and inhibited migration of GC [20]. The MYC-targeting siRNA might also be involved in the regulation of cell cycle and EMT.

The current study highlights the cell cycle regulation and non-coding RNA regulation in diffuse- and intestinal-type GC. A long non-coding RNA LINC00460 induced EMT and cell proliferation and metastasis in head and neck squamous cell carcinoma via translocation of peroxiredoxin-1 into the cell nucleus [21]. The non-coding RNAs, such as microRNAs, have various roles in biological and pathological processes, including cell-cycle, proliferation, EMT, and drug resistance [22]. The investigation in RNA regulation and EMT in the point of view of anti-cancer drug resistance and identification of therapeutic targets would be the future research direction.

5. Conclusions

The several canonical pathways have been found to be altered in diffuse- and intestinal-type GC. Canonical pathway on "Cell cycle: G_1/S checkpoint regulation" was activated in diffuse-type GC, and "Cell cycle control of chromosomal replication" pathway and "Cyclins and cell cycle regulation" pathway was activated in intestinal-type GC. Canonical pathway related to "Role of BRCA1 in DNA damage response" was activated in intestinal-type GC compared to diffuse-type GC. Some molecules such as SMAD4 in the pathway of "Cell cycle: G_1/S checkpoint regulation" are involved in regulation of EMT. Cell cycle regulation may also be altered in EMT conditions in diffuse-type GC. The findings of the study where the activation states in the pathways related to cell cycle regulation alter in diffuse- and intestinal-type GC would highlight the significance of the cell

cycle regulation in EMT. Precise mechanism where compartment molecules in cell cycle pathways regulate EMT and CSCs would be future investigation.

Author Contributions: Conceptualization, S.T. and H.S.; methodology, S.T.; software, S.T.; formal analysis, S.T.; investigation, S.T.; data curation, S.T., K.A. and H.S.; writing—original draft preparation, S.T.; writing—review and editing, S.T., S.Q. and H.C.; visualization, S.T.; supervision, S.T. and A.H.; project administration, S.T., K.A., H.Y. and H.S.; funding acquisition, S.T., S.Q., R.O. and A.H. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: In this research, the RefSeq data of diffuse- and intestinal-type GC publicly available in The Cancer Genome Atlas (TCGA) of The cBioPortal for Cancer Genomics database [9-11] in National Cancer Institute (NCI) Genomic Data Commons (GDC) Data Portal was analyzed [12]. From the publicly available data of stomach adenocarcinoma in TCGA (NCI, USA: https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) [10], intestinal- and diffuse-type GC data, which are noted as chromosomal instability (CIN) (n=223) and genomically stable (GS) (n=50), respectively, in TCGA Research Network publication, were compared [11].

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