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Urothelial Cancer

Methylation of a Panel of MicroRNA Genes Is a Novel Biomarker for Detection of Bladder Cancer

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Abstract

Background: Dysregulation of microRNAs (miRNAs) has been implicated in bladder cancer (BCa), although the mechanism is not fully understood.
Objective: We aimed to explore the involvement of epigenetic alteration of miRNA expression in BCa.
Design, setting, and participants: Two BCa cell lines (T24 and UM-UC-3) were treated with 5-aza-2′-deoxycytidine (5-aza-dC) and 4-phenylbutyric acid (PBA), after which their miRNA expression profiles were analyzed using a TaqMan array (Life Technologies, Carlsbad, CA, USA). Bisulfite pyrosequencing was used to assess miRNA gene methylation in 5 cancer cell lines, 83 primary tumors, and 120 preoperative and 47 postoperative urine samples.
Outcome measurements and statistical analysis: Receiver operating characteristic (ROC) curve analysis was used to assess the diagnostic performance of the miRNA gene panel.
Results and limitations: Of 664 miRNAs examined, 146 were upregulated by 5-aza-dC plus PBA. CpG islands were identified in the proximal upstream of 23 miRNA genes, and 12 of those were hypermethylated in cell lines. Among them, miR-137, miR-124-2, miR-124-3, and miR-9-3 were frequently and tumor-specifically methylated in primary cancers (miR-137: 68.7%; miR-124-2: 50.6%; miR-124-3: 65.1%; miR-9-3: 45.8%). Methylation of the same four miRNAs in urine specimens enabled BCa detection with 81% sensitivity and 89% specificity; the area under the ROC curve was 0.916. Ectopic expression of silenced miRNAs in BCa cells suppressed growth and cell invasion.
Conclusions: Our results indicate that epigenetic silencing of miRNA genes may be involved in the development of BCa and that methylation of miRNA genes could be a useful biomarker for cancer detection.
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1. Introduction

MicroRNAs (miRNAs) are a group of small noncoding RNAs that negatively regulate the translation and stability of partially complementary target mRNAs. In that way, they play important roles in a wide array of biologic processes, including cell proliferation, differentiation, and apoptosis [1]. Increasing evidence suggests that dysregulation of miRNA expression contributes to the initiation and progression of human cancer [2,3]. Altered miRNA expression is thought to play an important role in the pathogenesis of bladder cancer (BCa) and in certain tumor phenotypes. For instance, high-grade BCa exhibits upregulation of several miRNAs, including miR-21, which suppresses p53 function [4]. In addition, miR-21-to-miR-205 expression ratios are elevated in invasive BCa cells [5], while miR-200 family members regulate epithelial-to-mesenchymal transition by targeting transcription repressors ZEB1 and ZEB2 in BCa cells [6].

Although the mechanisms underlying miRNA dysregulation in cancer are not yet fully understood, recent studies have shown that the silencing of several miRNAs is tightly linked to epigenetic mechanisms, including histone modification and DNA methylation [7,8]. For example, treatment with a histone deacetylase (HDAC) inhibitor and a DNA methyltransferase (DNMT) inhibitor restored expression of various miRNAs in cancer cells [7,9], and the list of miRNA genes methylated in cancer is rapidly growing [10]. Studies have also shown that restoration of epigenetically silenced miRNAs may be an effective strategy for treating cancer and that aberrant methylation of miRNA genes could be a useful biomarker for cancer detection [10,11]. In addition, it was recently shown that the silencing of miRNA expression in BCa is associated with DNA methylation, often involving the CpG island (CGI) or CpG shore [12,13]. In an effort to identify novel biomarkers and treatment targets in BCa, we aimed to identify miRNAs epigenetically silenced in BCa cells by screening for miRNAs whose expression is upregulated by DNA demethylation and HDAC inhibition. We also investigated the methylation of miRNA genes in urine specimens and assessed its clinical usefulness as a biomarker for detection of BCa.

2. Materials and methods

2.1. *Cell lines and tissue samples*

BCa cell lines (T24, UM-UC-3, HT-1197, HT-1376, SW780, and 5637) and a normal urothelial cell line (SV-HUC-1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; Supplementary Table 1). A colorectal cancer cell line HCT116 harboring genetic disruptions within the DNMT1 and DNMT3B loci (DNMTs KO) have been described previously [8]. T24 and UM-UC-3 cells were treated first with 1 μM or 0.1 μM 5-aza-2′-deoxycytidine (5-aza-dC; Sigma-Aldrich, St Louis, MO, USA) for 72 h, and then with 3 mM 4-phenylbutyric acid (PBA; an HDAC inhibitor, Sigma-Aldrich) for 72 h, replacing the drug and medium every 24 h. A total of 83 primary BCa specimens were collected from patients who underwent radical cystectomy (RC) or transurethral resection of bladder tumor (TURBT; 66 males and 17 females; median age: 72 yr; range: 34–90 yr). Of the 83 patients, 73 underwent surgical resection after initial diagnosis, 7 received chemotherapy before surgery, and 3 are recurrent cases. Samples of nontumorous bladder tissue adjacent (<2 cm) to and distant (>2 cm) from the tumors were also collected. Six samples of normal urothelial tissue from renal cell carcinoma (RCC) patients who underwent nephrectomy were also collected. Informed consent was obtained from all patients before collection of the specimens, and this study was approved by the institutional review board. Total RNA was extracted using a *mir*Vana miRNA isolation kit (Life technologies, Carlsbad, CA, USA). Genomic DNA was extracted using the standard phenol-chloroform procedure.

2.2. *Urine samples*

Voided urine specimens were collected from 20 cancer-free individuals (Supplementary Table 2) and 86 BCa patients. In addition, postoperative voided urine samples were collected from 36 of the 86 patients 3–10 d after TURBT treatment. As an independent test set, preoperative urine samples were collected from 34 BCa patients, and postoperative samples were collected from 11 patients. The postoperative urine samples were collected from patients in whom tumors were successfully resected without leaving residual tumors. The urine (10 ml) was mixed with 5 ml of ThinPrep PreservCyt solution (Hologic, Bedford, MA, USA) and stored at 4 °C. Each sample was centrifuged at 3000 rpm for 10 min, and genomic DNA was extracted from the pelleted sediment using the standard phenol-chloroform procedure.

2.3. *MicroRNA expression profiling*

Expression of 664 miRNAs was analyzed using a TaqMan MicroRNA array v2.0 (Life Technologies). Briefly, 1 μg of total RNA was reverse-transcribed using a Megaplex Pools kit (Applied Biosystems, Foster City, CA, USA), after which miRNAs were amplified and detected using polymerase chain reaction (PCR) with specific primers and TaqMan probes. U48 snRNA (RNU48, Life Technologies) served as an endogenous control.

2.4. *Quantitative real-time polymerase chain reaction of miRNA*

Expression of selected miRNAs was analyzed using TaqMan microRNA assays. Briefly, 5 ng of total RNA were reverse-transcribed using specific stem-loop real-time primers, after which they were amplified and detected using PCR with specific primers and TaqMan probes. U6 snRNA (RNU6B, Life Technologies) served as an endogenous control.

2.5. *Methylation analysis*

Bisulfite conversion of genomic DNA, methylation-specific PCR (MSP), bisulfite sequencing, and bisulfite pyrosequencing were carried out as described previously [8]. Primer sequences and PCR product sizes are listed in Supplementary Table 3. Primer locations for methylation analysis are shown in Supplementary Figure 1.

2.6. *Transfection of microRNA precursor molecules*

BCa cells (1×10^6 cells) were transfected with 100 pmol of Pre-miR miRNA Precursor Molecules (Life Technologies) or Pre-miR miRNA Molecules Negative Control #1 using a Cell Line Nucleofector kit R (Lonza, Basel, Switzerland) with a Nucleofector I electroporation device (Lonza) according to the manufacturer's instructions. The viability of the miRNA precursor transfectants was analyzed using water-soluble tetrazolium salt (WST) assays [8]. Cell invasion was assessed using Matrigel invasion assays [8].

2.7. *Gene expression microarray analysis*

One-color microarray-based gene expression analysis was carried out according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). Briefly, 100 ng of total RNA were amplified and labeled using a Low-input Quick AmpLabeling Kit One-color (Agilent Technologies), after which the synthesized cRNA was hybridized to a SurePrint G3 Human GE microarray (G4851F; Agilent Technologies). The microarray data were then analyzed using GeneSpring GX version 11 (Agilent Technologies). The Gene Expression Omnibus accession number for the miRNA microarray data is GSE41760.

2.8. *Statistical analysis*

All data were analyzed using GraphPad Prism 4.0 statistical software (GraphPad Software, La Jolla, CA, USA). Quantitative variables were analyzed using a Student *t* test and one-way analysis of variance (ANOVA) with a post hoc Tukey test. Fisher exact test was used for analysis of categorical data. The Pearson correlation coefficient was used to evaluate correlations between continuous data. Receiver operating characteristic (ROC) curves for the diagnosis of BCa were constructed on the basis of the methylation levels, followed by calculation of the area under the curve (AUC). The best cut-off value for each miRNA gene was defined as the point on the ROC curve closest to the upper left corner. A diagnostic scoring system using urinary DNA methylation was constructed by analyzing the training set using the following three-step algorithm: (1) The methylation status of miRNA genes was assessed using the respective cut-off values; (2) the number of methylation-positive genes was determined, which we termed the *miR-methylation score* (M-score); and (3) the samples were classified into five groups based on the M-score. The value of $p < 0.05$ (two-sided) was regarded as significant.

3. Results

3.1. *Identification of epigenetically silenced microRNA genes in bladder cancer*

To identify epigenetically silenced miRNAs in BCa, we performed TaqMan array analysis using two BCa cell lines (T24 and UM-UC-3) treated with 1 μM 5-aza-dC plus 3 mM PBA. Of the 664 miRNAs examined, the drug treatment induced upregulation (more than five-fold) of 208 miRNAs in T24 cells and 200 miRNAs in UM-UC-3 cells. Of those, 146 miRNAs were upregulated in both cell lines (Supplementary Fig. 2 and 3; Supplementary Table 4). We selected 23 miRNA genes that harbored CGIs in the proximal upstream (<5 kb) of their coding regions (Supplementary Table 5), and subsequent MSP analysis revealed that the CGIs of 12 were hypermethylated in multiple BCa cell lines (Fig. 1A). These miRNAs were also induced by a low dose (0.1 μM) of 5-aza-dC plus PBA, making it unlikely that the observed induction was a secondary effect of DNA damage (Supplementary Fig. 4).

We next used bisulfite pyrosequencing to quantitatively analyze the methylation of the 12 miRNA genes showing CGI methylation in a series of BCa tissues ($n = 26$), a sample of normal urothelium tissue, and a normal urothelial cell line (SV-HUC-1). We found that four miRNA genes (miR-137, miR124-2, miR-124-3, and miR-9-3) were frequently methylated in primary tumors, though their methylation levels were limited in normal urothelium (Fig. 1B; Supplementary Fig. 5 and 6). In addition, we observed a marked

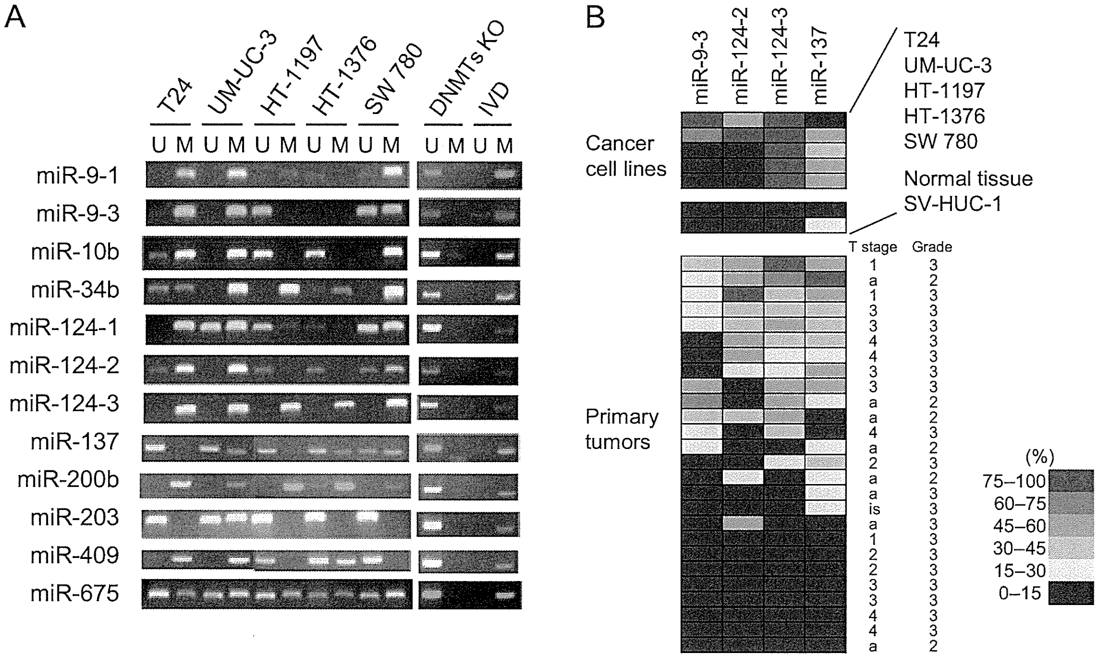

Fig. 1 – Methylation analysis of microRNA (miRNA) genes in bladder cancer (BCa). (A) Methylation-specific polymerase chain reaction (PCR) analysis of the CpG islands of 12 miRNA genes in the indicated cell lines. In vitro methylated DNA and DNA methyltransferase knockout cells served as positive and negative controls, respectively. Bands in the "M" lanes are PCR products obtained with methylation-specific primers; those in the "U" lanes are products obtained with unmethylated-specific primers. (B) Summarized results for the bisulfite pyrosequencing of miRNA genes in BCa cell lines, a sample of normal urothelial tissue, a normal urothelial cell line SV-HUC-1, and a set of primary BCa tissues ($n = 26$). Tumor stages and grades are indicated on the right.
DNMTs KO = DNA methyltransferase knockout cells; IVD = in vitro methylated DNA.

Table 1 – Correlation between microRNA gene methylation and the clinicopathologic features of bladder cancer

	($n = 83$)	miR-137 met (%)			miR-124-2 met (%)			miR-124-3 met (%)			miR-9-3 met (%)		
		Mean	SD	p^*	Mean	SD	p^*	Mean	SD	p^*	Mean	SD	p^*
Age, yr:													
Median (range)	72 (34–90)	–	–	0.394	–	–	0.277	–	–	0.147	–	–	0.065
Gender:													
Male	66	26.7	19.9	–	25.1	23.9	–	32.0	24.4	–	19.8	17.4	–
Female	17	34.6	22.1	0.160	22.2	11.1	0.630	31.8	20.6	0.974	17.3	13.0	0.591
T stage:													
Ta	35	31.3	23.4	–	25.1	22.1	–	29.7	25.2	–	21.0	18.5	–
Tis	4	23.5	19.7	–	24.6	28.8	–	22.5	15.4	–	12.7	4.4	–
T1	8	33.4	19.2	–	33.9	29.9	–	42.6	25.2	–	24.9	16.2	–
≥T2	36	24.9	17.8	0.486	21.8	19.3	0.575	32.7	22.1	0.458	17.0	15.3	0.463
Grade:													
1	1	15.3	–	–	1.9	–	–	5.8	–	–	11.2	–	–
2	27	29.3	23.4	–	21.3	18.9	–	28.6	24.6	–	21.3	16.6	–
3	55	28.1	19.3	0.795	26.5	23.2	0.360	34.1	22.9	0.331	18.4	16.8	0.676
LN metastasis:													
N0	73	28.6	21.0	–	24.9	22.4	–	32.4	24.0	–	20.1	17.3	–
N1–N3	10	26.7	17.8	0.782	21.8	18.2	0.685	28.3	20.5	0.605	13.3	7.3	0.231

SD = standard deviation; LN = lymph node; ANOVA = analysis of variance.

[*] Pearson correlation coefficient, student *t* test, or ANOVA.

reduction in the methylation levels in BCa cells treated with 5-aza-dC plus PBA, which is consistent with the upregulation of miRNAs (Supplementary Fig. 7).

3.2. *Methylation of microRNA genes in primary bladder cancer*

We next examined the methylation levels of miR-137, miR-124-2, miR-124-3, and miR-9-3 in a larger set of primary tumors ($n = 83$), along with adjacent and distant nontumorous bladder tissues from the same patients (Table 1). Elevated levels of miRNA gene methylation (>15.0%) were frequently detected in primary BCa tissues (miR-137: 57 of 83, 68.7%; miR-124-2: 42 of 83, 50.6%; miR-124-3: 54 of 83, 65.1%; miR-9-3: 38 of 83, 45.8%), and the tumor tissues exhibited significantly higher methylation levels than their nontumorous counterparts (Fig. 2A). In addition, we found that levels of miRNA gene methylation were more frequently elevated in adjacent nontumorous bladder tissues (AN; miR-137: 26 of 74, 35.1%; miR-124-2: 19 of 74, 25.7%; miR-124-3: 15 of 74, 20.3%; miR-9-3: 12 of 74, 16.2%) than in more distant nontumorous tissues (DN; miR-137: 18 of 83, 21.7%; miR-124-2: 6 of 83, 7.2%; miR-124-3: 11 of 83, 13.3%; miR-9-3: 9 of 83, 10.8%). No significant correlation was found between the levels of miRNA gene methylation and the clinicopathologic characteristics of the patients (Table 1).

When we examined the methylation status of miR-137 in selected tissue specimens in more detail, we observed dense methylation in tumor tissues but only scattered methylation in nontumorous tissues (Fig. 2B). We then compared the levels of miR-137 expression determined in TaqMan assays with the methylation levels obtained by bisulfite pyrosequencing in selected pairs of tumors and corresponding distant nontumorous tissues (Fig. 2C). We found that there was an inverse relationship between the expression of miR-137 and its methylation, which suggests that CGI methylation is associated with the downregulation of miR-137 in BCa tissues.

3.3. *Detection of microRNA gene methylation in urine samples*

To assess the usefulness of miRNA gene methylation, we collected voided urine specimens from 86 BCa patients (Table 2) and 20 cancer-free individuals. Upon performing bisulfite pyrosequencing, we observed elevated methylation of miR-137, miR-124-2, miR-124-3, and miR-9-3 in the urine samples from the cancer patients (Fig. 3A) but only limited methylation of the genes in cancer-free individuals (Fig. 3B). Moreover, the methylation levels in the urine samples correlated positively with those in the corresponding tumor tissues (Supplementary Fig. 8). Notably, when we then collected postoperative voided urine samples from 36 of the 86 patients after surgical resection of their tumors, we observed dramatically reduced methylation levels (Fig. 3A; Supplementary Fig. 9).

To further evaluate the clinical usefulness of the miRNA gene methylation in urine samples, we carried out ROC curve analysis to assess its ability to distinguish preoperative from postoperative samples (Fig. 3C). The most discriminating cut-offs for miR-137, miR-124-2, miR-124-3, and miR-9-3 were 5.2% (sensitivity, 77.9%; specificity, 77.8%), 5.2% (sensitivity, 69.8%; specificity, 88.9%), 12.0% (sensitivity, 65.1%; specificity, 97.2%), and 7.2% (sensitivity, 69.4%; specificity, 86.1%), respectively (Table 3). We next compared these results with those obtained with urine cytology. Based on the urinary cytology using Papanicolaou's classification of the 86 patients, 55 (64%) were diagnosed as class I or II, 15 (17%) were class III, and only 16 (19%) were class IV or V (strongly suggestive or conclusive of malignancy), suggesting that the sensitivity of urinary methylation for detection of BCa is significantly greater than that of conventional cytology (Supplementary Table 6).

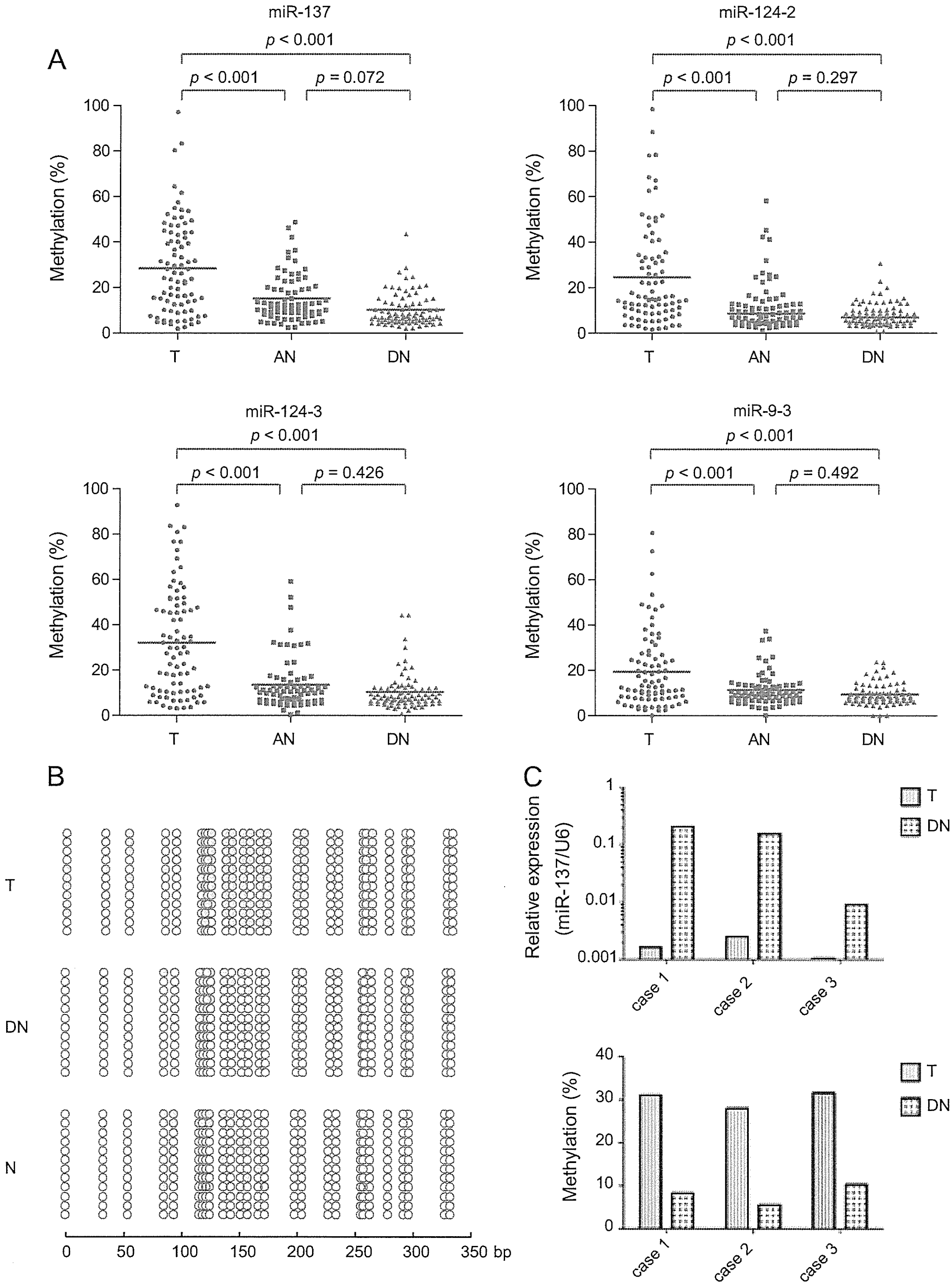

Fig. 2 – **Analysis of microRNA (miRNA) gene methylation in primary bladder cancer. (A) Summarized results of bisulfite pyrosequencing of the indicated miRNA genes in primary tumors (T; $n = 83$), nontumorous bladder tissues adjacent to the tumors (AN; $n = 74$), and nontumorous bladder tissues distant from the tumors (DN; $n = 83$). $p < 0.05$. (B) Bisulfite sequencing analysis of the miR-137 CpG island (CGI) in a pair of tumor (T) and distant nontumorous tissues (DN). (C) Inverse relationship between the expression and methylation of miR-137 in three pairs of tumor (T) and distant nontumorous tissues (DN). Expression was assessed in TaqMan assays (upper panel), and methylation was determined by bisulfite pyrosequencing (lower panel).**

To develop a more efficient diagnostic method for detecting BCa, we constructed a scoring system using the urinary methylation of the four methylated miRNA genes (Fig. 4). Using the cut-off value for each gene (Table 3), we classified the samples into five groups based on the M-score. A ROC curve was then constructed to evaluate the ability of the scoring system to distinguish preoperative from postoperative urine samples by plotting the sensitivity over 1-specificity at each point (Fig. 4B). We then validated the diagnostic system by analyzing an independent test set

Table 2 – Clinicopathologic characteristics of the patients in the training and test sets

	Training set ($n = 86$)	Test set ($n = 34$)
Age, yr:		
Median (range)	73 (42–90)	71 (58–93)
Gender, no.:		
Male	69	25
Female	17	9
T stage, no.:		
Ta	34	16
Tis	7	5
T1	12	8
≥T2	33	5
Grade, no.:		
1	1	0
2	28	14
3	57	20
Lymph node metastasis, no.:		
N0	81	32
N1–N3	5	2
Treatment, no.:		
TURBT	64	30
RC	22	4

TURBT = transurethral resection of bladder tumor; RC = radical cystectomy.

(Table 2). AUCs in both sets were high (training set: 0.916; test set: 0.910), confirming the accuracy of our system for detecting BCa using urinary miRNA gene methylation (Fig. 4). We also found that our scoring system could effectively detect early-stage Ta and low-grade (grades 1 and 2) BCa (sensitivity: 0.679; specificity: 0.889; AUC = 0.862), which was undetectable using urinary cytology (Supplementary Fig. 10).

3.4. *Functional analysis of microRNAs*

To test whether any of the miRNAs could act as tumor suppressors, we transfected BCa cells with an miRNA precursor molecule or a negative control, and then carried out cell viability assays. The assays showed that ectopic expression of miR-137 or miR-124 suppressed BCa cell proliferation, whereas miR-9 exerted no significant suppressive effect on growth (Supplementary Fig. 11 and 12). We then carried out Matrigel invasion assays to test the effect of the miRNAs on cell invasion. Although we detected no effect of miR-137 and miR-124 on cell invasion, ectopic expression of miR-9 suppressed the invasiveness of BCa cells (Supplementary Fig. 13).

Finally, to further clarify the effect of miRNAs, we carried out a gene expression microarray analysis of SW780 cells transfected with a miR-137 precursor or a negative control. We found that 1326 probe sets (1016 unique genes) were downregulated (more than two-fold) by ectopic miR-137 expression, including the previously reported miR-137 target genes cyclin-dependent kinase 6 (*CDK6*), cell division cycle 42 (*CDC42*), and aurora kinase A (*AURKA*) [14,15]. Among the 1016 downregulated genes, the TargetScan program predicted that 144 genes are potential targets of miR-137 (Supplementary Table 7). Moreover, Gene Ontology analysis revealed that genes related to the cell cycle were significantly enriched among the affected genes (Supplementary Table 8). Our results strongly suggest that the miRNAs in question act as tumor suppressors in BCa.

4. Discussion

We identified four miRNA genes (miR-137, miR-124-2, miR-124-3, and miR-9-3) that were frequently methylated in both cultured and primary BCa cells. Earlier studies have shown that these miRNAs are tumor-suppressive or tumor-related and that they are epigenetically silenced in cancers of various origins. Hypermethylation of miR-137 was first discovered in oral cancer [16] and has since been noted in other malignancies, including cancers of the colon [14] and stomach [3]. Within cancer cells, miR-137 targets *CDK6*, *CDC42*, and *AURKA*, which is indicative of its tumor-suppressive properties [14–16], whereas in normal cells, miR-137 regulates neuronal differentiation through targeting enhancer of zeste homolog 2 (*EZH2*) and mindbomb E3 ubiquitin protein ligase 1 (*MIB1*) [17,18]. Methylation of miR-124 family genes (miR-124-1, miR-124-2, and miR-124-3) was identified in colorectal cancer [19] and was also

Table 3 – Receiver operating characteristic analysis of microRNA gene methylation to detect bladder cancer

Gene name	Cut-off, %	Training set		
		AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
miR-137	5.2	0.782 (0.701–0.862)	77.91 (67.67–86.14)	77.78 (60.85–89.88)
miR-124-2	5.2	0.769 (0.686–0.851)	69.77 (58.92–79.21)	88.89 (73.94–96.89)
miR-124-3	12.0	0.805 (0.730–0.880)	65.12 (54.08–75.08)	97.22 (85.47–99.93)
miR-9-3	7.2	0.778 (0.697–0.860)	69.41 (58.47–78.95)	86.11 (70.50–95.33)

Gene name	Cut-off, %	Test set		
		AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
miR-137	5.2	0.816 (0.693–0.938)	79.41 (62.10–91.30)	63.64 (30.79–89.07)
miR-124-2	5.2	0.866 (0.758–0.975)	79.41 (62.10–91.30)	90.91 (58.72–99.77)
miR-124-3	12.0	0.901 (0.807–0.995)	58.82 (40.70–75.35)	100.0 (71.51–100.0)
miR-9-3	7.2	0.797 (0.660–0.934)	76.47 (58.83–89.25)	72.73 (39.03–93.98)

AUC = area under the curve; CI = confidence interval.

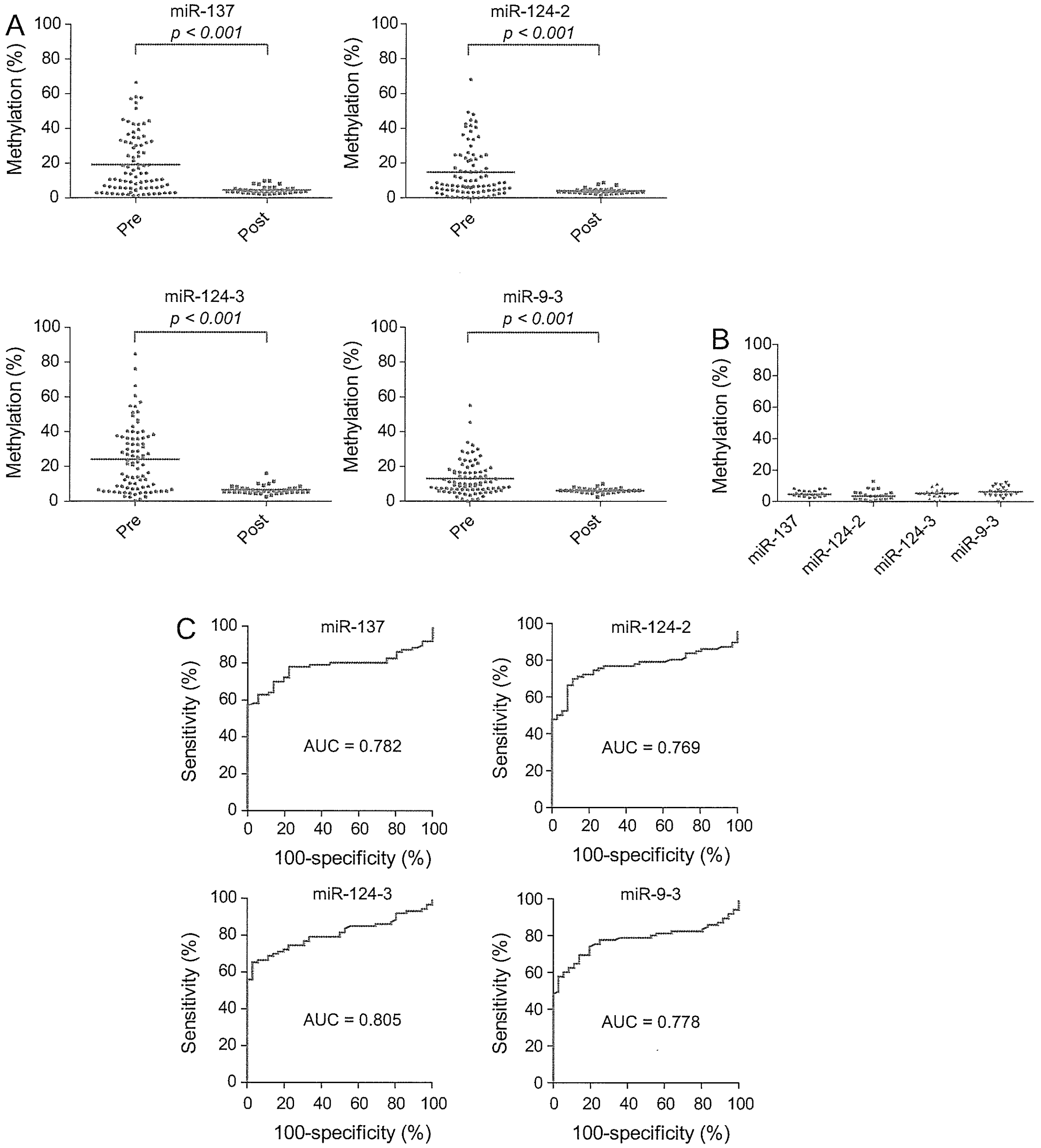

Fig. 3 – Detection of microRNA (miRNA) gene methylation in urine specimens from bladder cancer (BCa) patients. (A) Summary of bisulfite pyrosequencing analysis of the indicated miRNA genes in voided urine samples collected from BCa patients before (Pre: $n = 86$) and after surgical treatment (Post: $n = 36$). $p < 0.001$. (B) Bisulfite pyrosequencing results for miR-137, miR-124-2, miR-124-3, and miR-9-3 in voided urine samples from cancer-free individuals ($n = 20$). (C) Receiver operating characteristics curve analysis of the ability of miRNA gene methylation to distinguish preoperative and postoperative urine samples.
AUC = area under the curve.

found in gastric cancer [20], hematologic malignancies [21], and hepatocellular carcinoma [22]. In addition, screening for methylated miRNA genes in metastatic cancer cell lines also identified miR-9 family genes (miR-9-1, miR-9-2, and miR-9-3) [23].

Cumulative evidence suggests that miRNAs play important roles in the pathogenesis of BCa, and previous studies demonstrated their epigenetic silencing in the disease. For example, miR-34a, which is a direct target of p53 and a candidate tumor suppressor gene, is frequently methylated and silenced in many types of cancer, including BCa [24]. In addition, Wiklund et al. found that the silencing of miR-200 family genes and miR-205 is associated with DNA methylation in invasive BCa [12]. They also showed that reduced expression of miR-200c is associated with disease progression and poor outcome, suggesting that epigenetic silencing of miR-200 family genes could be a prognostic marker in BCa. Recently, Dudziec et al. carried out an miRNA microarray analysis after treating normal urothelium and urothelial cancer cell lines with 5-azacytidine. They identified 4 mirtrons and 16 miRNAs whose silencing was associated with DNA methylation [13]. Some of those

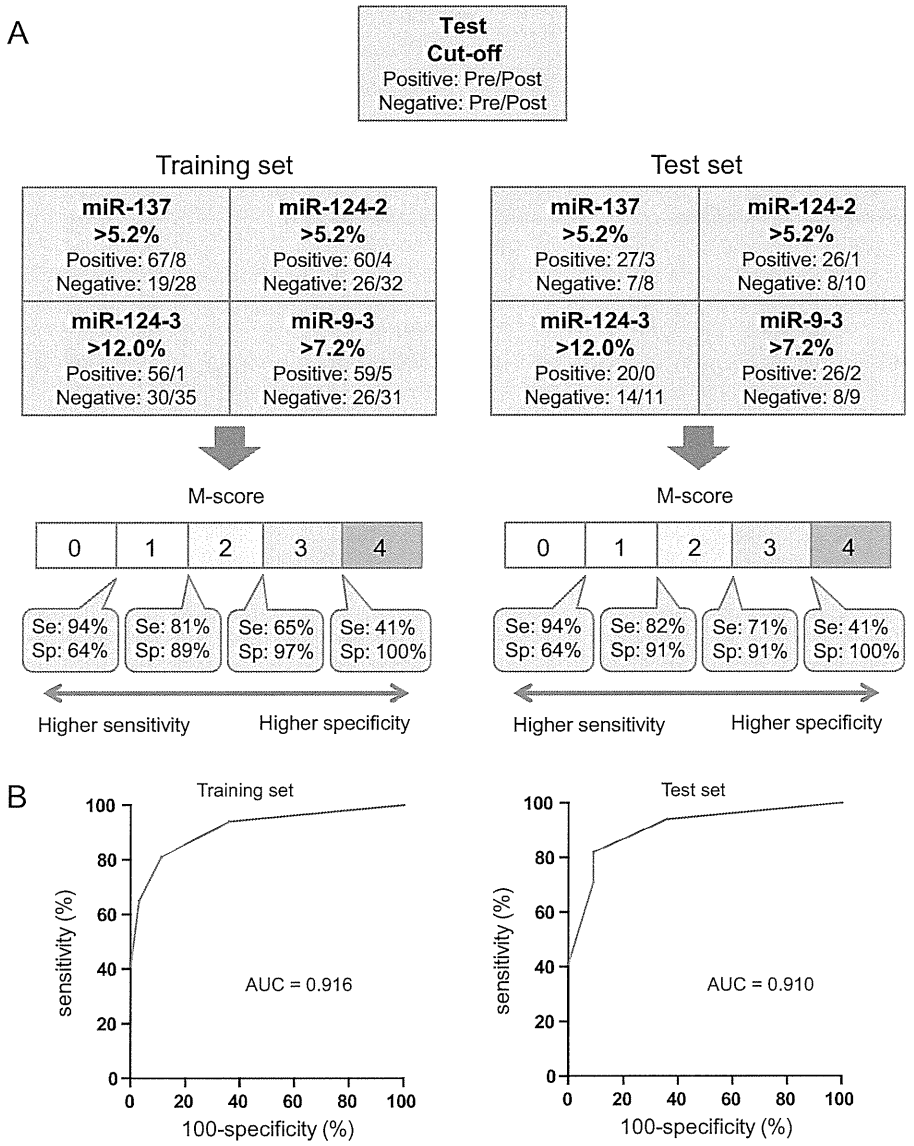
A
Test
Cut-off
Positive: Pre/Post
Negative: Pre/Post
Training set
miR-137 >5.2% Positive: 67/8 Negative: 19/28
miR-124-2 >5.2% Positive: 60/4 Negative: 26/32
miR-124-3 >12.0% Positive: 56/1 Negative: 30/35
miR-9-3 >7.2% Positive: 59/5 Negative: 26/31
Test set
miR-137 >5.2% Positive: 27/3 Negative: 7/8
miR-124-2 >5.2% Positive: 26/1 Negative: 8/10
miR-124-3 >12.0% Positive: 20/0 Negative: 14/11
miR-9-3 >7.2% Positive: 26/2 Negative: 8/9
M-score
0 1 2 3 4
Se: 94% Sp: 64%
Se: 81% Sp: 89%
Se: 65% Sp: 97%
Se: 41% Sp: 100%
Se: 94% Sp: 64%
Se: 82% Sp: 91%
Se: 71% Sp: 91%
Se: 41% Sp: 100%
Higher sensitivity
Higher specificity
B
Training set
Test set
sensitivity (%)
100-specificity (%)
0 20 40 60 80 100
AUC = 0.916
AUC = 0.910

Fig. 4 – Diagnostic system for detecting bladder cancer (BCa) using urinary microRNA (miRNA) gene methylation. (A) Workflow of a system established based on the ability to distinguish preoperative from postoperative urine. Results of the training set are shown on the left; those of test set are on the right. The methylation status of miRNA genes in preoperative (training set: $n = 86$; test set: $n = 36$) and postoperative urine (training set: $n = 34$; test set: $n = 11$) was determined using the cut-off values in the respective boxes. A miR-methylation score (M-score) was determined from the number of methylation-positive genes, and samples were classified into five groups based on the M-score. The sensitivity (Se) and specificity (Sp) at each point are indicated below. (B) Receiver operating characteristic curve analysis of the training and test sets. Areas under the curve are shown in the graph. M-score = miR-methylation score; AUC = area under the curve.

mirtrons and miRNAs, including miR-9 family genes, more frequently exhibited CpG shore methylation than CGI methylation, suggesting that methylation in both the CpG shore and CGI is related to epigenetic silencing of miRNA in BCa. Interestingly, miR-9-1 and -9-2 were associated with both CGI and CpG shore methylation, whereas miR-9-3 showed only CGI methylation [13]. Consistent with those findings, we observed that among the miR-9 family genes, miR-9-3 most frequently showed CGI methylation.

Methylation of several miRNA genes is strongly related to the clinical characteristics of cancer, suggesting its potential usefulness as a biomarker. For instance, methylation of miR-9-1 and -9-3 is reportedly associated with metastatic recurrence of RCC, which is indicative of the possible role of miR-9 in cancer metastasis [25]. Despite this report, however, we did not find a significant difference in the levels of miR-9-3 methylation between noninvasive and invasive BCa tissues. Further study to clarify the functions of these miRNAs in BCa will be needed.

Recent studies have shown that miRNA levels in urine could serve as a molecular marker for detection of BCa. For instance, expression of miR-96 and miR-183 is reportedly upregulated in urothelial cancer, and their detection in urine strongly distinguished cancer patients from cancer-free patients [26]. Miah et al. also showed that evaluation of a panel of 10 miRNAs in urine is a highly sensitive method of detecting BCa [27]. DNA methylation is another potential molecular marker detectable in urine specimens. Several protein-coding genes are targets of DNA methylation in BCa, and their urinary methylation appears to be a useful biomarker [28,29]. For instance, methylation of 11 protein-coding genes found in urine sediments revealed the presence of BCa with a high sensitivity and specificity [30], and in another study a panel of three genes (growth differentiation factor 15 [*GDF15*], transmembrane protein with EGF-like and two follistatin-like domains 2 [*TMEFF2*], and vimentin [*VIM*]) in urine could be used to accurately detect BCa [31]. In the present study, we show for the first time that methylation of miRNA genes could serve as a biomarker for detection of BCa. Methylation of miRNA genes was readily detectable in voided urine from cancer patients, and its levels were dramatically reduced after tumor resection, confirming its tumor specificity. We also showed that a combination of multiple miRNA genes could accurately distinguish between preoperative and postoperative urine samples.

Our study has several limitations. The prognostic value of miRNA gene methylation remains unclear, because the prognosis of the patients in this study is not yet available. A follow-up study in post-treatment patients will be needed to test whether urinary methylation can predict outcome or detect BCa recurrence. In addition, urinary methylation in non-BCa patients (eg, patients with other types of cancer) should be tested to evaluate the specificity of our method. Further studies to address these issues would contribute to overcoming the difficulties in translating our present findings into clinical practice.

5. Conclusions

We identified four miRNA genes that are frequent targets of epigenetic silencing in BCa. Although their specific functions in bladder carcinogenesis remain unknown, it is evident that restoration of these miRNAs may be an effective anticancer therapy. Furthermore, methylation of these miRNA genes in urine specimens could serve as a useful and noninvasive biomarker for accurate detection of BCa.

Author contributions: Hiromu Suzuki had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition of data: Shimizu, Ashida, Hatahira, Yamamoto, Maruyama, Kai.

Analysis and interpretation of data: Shimizu, Suzuki, Nojima.

Drafting of the manuscript: Shimizu, Suzuki.

Critical revision of the manuscript for important intellectual content: Taiji Tsukamoto.

Statistical analysis: Nojima.

Obtaining funding: Suzuki, Toyota, Tsukamoto.

Administrative, technical, or material support: Kitamura, Masumori, Tokino, Imai, Tsukamoto.

Supervision: Minoru Toyota, Taiji Tsukamoto.

Other (specify): None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2012.11.030.

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Review Article Open Access

New Paradigm of T cell Signaling: Learning from Malignancies

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Abstract

T cells are key mediators of cell-mediated immunity. Their functions and proliferation result from T cell-specific receptor signaling (TCR/CD28) that activates the NF-κB, NFAT, Ras-MAPK, and PI3K-Akt pathways. Their development and activation also involve a complex array of signaling pathways that regulate gene expression networks, including signaling of mTOR, Notch, Wnt, Hedgehog, TGF-β, and toll-like receptors. Furthermore, recent discoveries have provided two molecular hallmarks of potential generality: miRNA patterns and polycomb-mediated epigenetic reprogramming, which can strongly coordinate the balance between molecular networks in lymphocytes. Their deregulation apparently causes T cell disorders, such as T cell acute lymphoblastic leukemia (T-ALL), and human T cell leukemia virus (HTLV-1)-induced adult T cell leukemia (ATL). This review continues with a description of our understanding of crosstalk among the signaling pathways, which contribute to the highly orchestrated development of T cell fate specification under both normal physiological and pathological conditions.

Introduction

T cells use diverse genetic programs to direct the development of distinct lineages and the generation of several effector functions required for innate and adaptive immunity. The molecular networks in T cells are strictly controlled by the input from intercellular and extracellular signals. T cell activation is basically achieved by antigen-presenting cells (APCs). Antigen recognition by and signaling through the T cell receptor (TCR) are crucial processes for T cells and provide the molecular underpinning for the specificity and execution of immune system responses.

TCR-mediated signaling pathways are the bare bones of T cell activation. However, several other signaling pathways are intimately associated with T cell development, activation, and homeostasis. In addition, newly emerging molecular characteristics have been proposed on the basis of studies of normal T cells and T cell malignancies. This new paradigm includes miRNA-mediated gene regulation and epigenetic reprogramming.

In this review, by dissecting each signaling pathway, we summarize the molecular hallmarks of T cells. We first briefly introduce the fundamental molecular mechanisms involved in T cell activation and gene expression related to T cell functions [1,2]. These signaling pathways are also involved in T cell development and disorders. We next discuss the newly emerging signaling pathways involved in T cell biology. Genetic and physiological studies have indicated that the pathways primarily identified in the areas of development, regeneration, and innate immunity are also closely associated with T cell functions and their appropriate development. Finally, we discuss regarding a conceptual advance, i.e., crosstalk between several signaling pathways.

The complexity of signaling networks within T cells confers robustness to specific and diverse gene expressions and biological functions. The complex signaling regulation that is involved within different environments has been suggested from investigations in immature and mature T cell malignancies, including T cell acute lymphoblastic leukemia (T-ALL) and adult T cell leukemia/lymphoma (ATL).

Proximal Signaling of TCR-mediated T cell Activation

T cells are instructed to use their developmental and effector programs through stimulation of the TCR complex and costimulatory molecules, which are presented by professional APCs, e.g., macrophages, dendritic cells, and B cells. TCR signaling is initiated by CD3 phosphorylation. The CD3 proteins comprise a series of dimers, including γε, δε, and ζζ, which are associated with a single TCRαβ heterodimer (Figure 1). After the recognizing cognate complexes of foreign peptide and self major histocompatibility complex (MHC) class II (pMHC) molecules, TCR/CD3 affects signaling cascades, including phosphorylation of proximal TCR components, Ras–mitogen-activated protein kinase (MAPK) signaling, activation of nuclear factor κB (NF-κB) by protein kinase C θ (PKCθ), and Ca^{2+} flux-mediated signaling [1]. In response to stimulation, signaling through these mediators is integrated with input from other signaling pathways. This provides the biological output after TCR recognition of a ligand.

CD3 phosphorylation is regulated by the local balance of the tyrosine kinase Lck, which is associated with the monomorphic coreceptors CD4 and CD8 and phosphatases in the TCR signaling complex. Functional key motifs within CD3ε, γ, δ, and ζ chains, designated immunoreceptor tyrosine-based activation motifs (ITAMs), are phosphorylated by Lck after TCR ligation, which is an early, prerequisite step for TCR-directed T cell activation. Importantly, conformational changes in CD3 molecules are directly induced by CD3 phosphorylation after pMHC–TCR ligation. These conformational changes in CD3ε and CD3ζ make ITAMs more accessible for phosphorylation and are required for efficient T cell activation.

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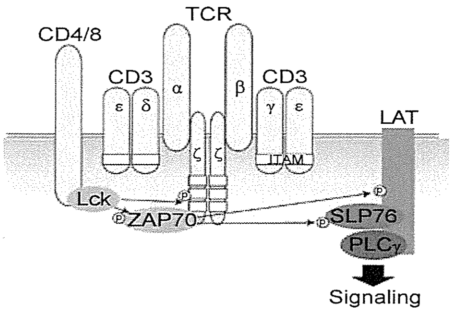

Figure 1: TCR/CD3-mediated proximal signaling. After engagement of TCR/CD3 complex, conformation change and following phosphorylation cascade is induced. Activated PLCγ coordinates the signaling required for T cell activation.

Phosphorylated CD3ζ (and other ITAM-containing proteins) provides recruitment sites of the 70-kDa phosphoprotein ZAP-70, a Syk kinase family member. That is, TCR engagement by pMHC leads to the activation of Src family PTK, such as Lck, which results in ITAM phosphorylation and ZAP-70 recruitment (Figure 1). The tandem SH2 domains of ZAP-70 are engaged by the phosphorylated ITAMs of CD3ζ, where in turn ZAP-70 is activated by Lck-mediated phosphorylation.

Among the most important ZAP-70 targets are the transmembrane adapter protein linker for the activation of T cells (LAT) and the cytosolic adapter protein Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76). Phosphorylated LAT in turn serves as a docking site to which a number of signaling proteins can bind. These two adapters form the backbone of a complex that includes several effector molecules to allow for the activation of multiple signaling pathways. Proteins that are incorporated in these assemblies include other scaffold molecules, such as Grb2, Gads, and enzymes like phospholipase C γ1 (PLCγ1), and phosphoinositide 3-kinase (PI3K). The coordination of these interactions and the ensuing signaling result in a stable, but dynamic, zone of contact between APCs and T cells; this zone has been designated the immunological synapse (IS). IS has become a paradigm for studying the signals exchanged between the two cell types.

A lipid raft is a microdomain within the plasma membrane that is rich in cholesterol, glycosphingolipids, and sphingomyelin. Lipid rafts accumulate at IS. Initial TCR activation and an early phosphorylation cascade precedes the formation of IS, which not only provides the sustained signaling required for gene regulation in T cells but may also control the eventual halt of the signaling pathways. It is important that the ability of a kinase to support TCR signaling critically depend on its lipid modification. Indeed, the accumulation of lipid rafts and Lck in these areas can accelerate increased phosphorylation. Within a lipid raft, the signaling backbone established by LAT and SLP-76 coordinates downstream signaling by controlling PLCγ1 activity. However, a large amount of data suggests that the formation of this complex is more complicated. For its optimal activity, PLCγ1 directly binds to SLP-76, LAT, and Vav1 as well as to its activating kinase Itk. Lck, which binds to CD4/8 and activates ZAP-70, also phosphorylates Itk. Itk in turn phosphorylates PLCγ1. Activated PLCγ1 then hydrolyzes the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2), producing the second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG), that are essential for T cell function (Figure 2).

TCR-mediated Gene Regulation via NFAT, NF-κB, and MAPK Cascades

TCR engagement results in the induction of expression of numerous genes required for T cell activation by triggering several signaling pathways. These pathways are described follows. Each of them acts specifically, but collaboratively, during T cell activation process.

Ras–MAPK pathway

TCR-induced DAG production results in the activation of two major key molecules: Ras and PKCθ. DAG recruits both RasGRP and PKCθ to the plasma membrane. Activated Ras, a guanine nucleotide-binding protein, initiates the MAPK phosphorylation and activation cascade by activating of the serine/threonine kinase Raf1 [1]. Raf1 is a MAPK kinase kinase (MAPKKK) that phosphorylates and activates MAPK kinases (MAPKKs), including MEK. This in turn phosphorylates and activates MAPK extracellular signal-regulated kinase 1 (Erk1) and Erk2 (Figure 2) [3]. Erk kinase activity is regulated by its phosphorylation. The transcription factor Elk1 is one of downstream molecule of Erk1 and induces Fos expression. Consequently, the activation of the activator protein-1 (AP1) complex constituted by Jun and Fos is sustained by the DAG–Ras pathway. Jun is activated via the Vav1–Rac pathway. In addition, Erk activation results in phosphorylation of signal transducer and activator of transcription 3 (STAT3) and Lck.

PKCθ-dependent NF-κB and AP1 pathways

Increased cellular concentrations of DAG also activates NF-κB signaling through PKCθ, a PKC family member that contains a lipid-binding domain specific for DAG. This domain is required for recruiting PKCθ to the lipid raft after TCR engagement. Moreover, Lck-mediated PKCθ phosphorylation appears to contribute to conformational changes required for binding of PKCθ to the lipid membrane. This concentration of PKCθ within the lipid raft enhances binding of PKCθ to DAG and results in PKCθ activation.

NF-κB plays its most important and evolutionarily conserved role in the immune system by regulating genes involved in inflammatory and immune responses as well as in some aspects of cell growth, survival and differentiation [4]. Both canonical (classical) and noncanonical (alternative) activation of this pathway in T cells are intimately involved in TCR-mediated T cell activation. PKCθ-mediated NF-κB induction has been shown to be selectively mediated by IKKβ that is

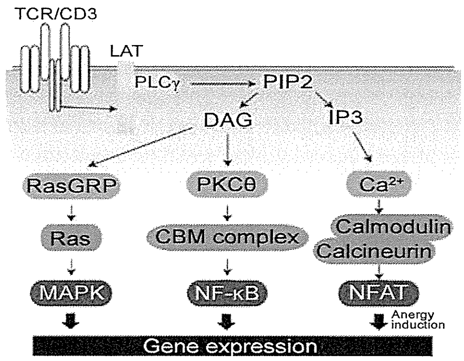

Figure 2: TCR-mediated signaling cascades. Engagement of TCR consequently induces expression of numerous genes required for T cell activation by triggering several signaling pathways, including Ras–MAPK pathway, NF-κB pathway, and NFAT pathway. Each of them specifically but collaboratively acts during T cell activation or anergy induction processes.

associated only with the canonical pathway, whereas noncanonical activation in T cells remains elusive. One study showed that MAP3K14 (also called NF-κB-inducing kinase; NIK), which is an essential factor for the noncanonical NF-κB cascade, was required for complete T cell activation [5].

Following TCR stimulation, PKCθ regulates the assembly of a CBM complex (CARMA1/Bcl10/MALT1) through its phosphorylation of CARMA1, which is required for CARMA1 oligomerization and association with Bcl10 [6]. MALT1 physically binds to Bcl10 and induces polyubiquitination of IKKγ, the regulatory subunit of the IKK complex, via activation of the E3 ubiquitin ligase TRAF6. K63 polyubiquitination appears to have a role in IKKγ activation, which results in IκB phosphorylation by IKK catalytic subunits and subsequent IκB degradation. Nuclear localization of NF-κB heterodimers induces gene activation (Figure 2). NF-κB activation is also regulated by costimulatory signaling (described below).

The original analysis of PKCθ knockout mice revealed that two transcription factors, NF-κB and AP1, are targets of PKCθ in TCR/CD28-costimulated T cells [7-9]. The pathway leading from PKCθ to AP1 activation is less clearly understood. One study has reported that SPAK, a Ste20-related MAP3K, is a direct substrate of PKCθ in the pathway leading to AP1, but not NF-κB, activation [10]. SPAK is most likely a mediator of PKCθ signals leading to AP1 activation, but intermediates downstream of SPAK in AP1 activation remain to be identified and functionally characterized.

Ca^{2+}-dependent NFAT pathway

PLCγ1-mediated IP3 generation stimulates Ca^{2+} permeable ion channel receptors (IP3R) located on the endoplasmic reticulum (ER). This leads to the release of ER Ca^{2+} stores into the cytoplasm. Because Ca^{2+} ions are universal second messengers in eukaryotic cells, TCR-induced increases in intracellular Ca^{2+} levels result in the activation of Ca^{2+}- and calmodulin-dependent transcription factors, including phosphatase calcineurin and the Ca^{2+}/calmodulin-dependent kinase (CaMK). Activated calcineurin then dephosphorylates members of the nuclear factor of activated T cells (NFAT) family, which results in their translocation to the nucleus. In the nucleus, NFATs form cooperative complexes with various other transcription factors, and this results in differential gene expression patterns and functional outcomes, depending on the context in which the TCR signal is delivered [11]. AP1 proteins are the main transcriptional partners of NFAT during T cell activation. Cooperation between NFAT and AP1 integrates two of the main signaling pathways i.e. the Ca^{2+} signaling and RAS-MAPK pathway. The NFAT-AP1 cooperation during T cell activation is responsible for a specific pattern of gene expression, which induces the functional changes that characterize an activated T cell. In the absence of AP1, NFAT proteins activate a distinct program of gene expression. The products of these genes inhibit T cell function at different levels and induce a status of T cell unresponsiveness, which is one of processes to induce the immune tolerance [12]. Thus, NFAT proteins control two opposing aspects of T cell function i.e. activation and anergy [13] (Figures 2 and 3).

Costimulation Signaling

A most important gene induced by TCR-mediated signaling is *IL-2*, which can activate STAT pathways. However, full induction of IL-2 expression is not achieved by TCR signaling alone, i.e., a nonresponsive state (anergy) exists in which T cells are refractory to restimulation. Additional engagement of other cell surface receptors provides and integrates signals required for anergy avoidance and productive T cell activation. Although many cell surface receptors can participate in costimulation signaling, CD28 provides more robust signals than other costimulatory molecules.

Weak TCR ligation does not lead to cell proliferation and differentiation; rather, it results in anergy or cell death. However, if engaged, CD28 strongly amplifies a weak TCR signal. In contrast, triggering of CD28 alone results in the transient expression of only a few genes and has no obvious biological consequences. A question has arisen whether TCR and the coreceptors induce separate signaling pathways (qualitative model) or whether the signaling pathways triggered by both receptor systems are entirely overlapping (quantitative model) [14].

Many studies have indicated that the signals generated by CD28 ligation have no unique effects. A microarray study showed that TCR-induced expression of thousands of genes in primary T cells was amplified (or suppressed) to varying degrees by CD28 costimulation, but that no new gene was induced by CD28 costimulation [15]. Indeed, all of the factors identified as components of the CD28 signaling pathway are those that are implicated in TCR-mediated signaling. These include PI3K, the Tec family of PTKs, such as Itk, Vav1, and the serine/threonine kinase Akt (also known as PKB), and NF-κB signaling.

CD28-mediated PI3K–Akt pathway

PI3K recruitment is a key event for coupling CD28 to several signaling pathways. Following engagement of CD28 with its ligands CD80 or CD86 expressed on APCs, the p85 regulatory subunit of PI3K associates with the phosphorylated cytoplasmic tail of CD28. This regulatory subunit recruits the p110 catalytic subunit of PI3K, which can convert PIP2 to PIP3 in the cell membrane. Then, the locally generated PIP3 serves as a docking site for the PH domains of PDK1 and its target Akt (Figure 3).

Akt phosphorylates multiple proteins involved in numerous cellular responses. Activated Akt enhances the nuclear translocation of NF-κB by associating with CARMA1 and facilitating the assembly of the CBM complex, a step critical for NF-κB activation [16]. In addition, one well-known Akt target is GSK-3, a serine/threonine kinase that influences the nuclear export of NFAT as well as the Wnt pathway. Thus, Akt-mediated GSK-3 inactivation might be a pathway responsible for prolonged NFAT nuclear localization and thus *IL-2* transcription following CD28 costimulation (Figure 3).

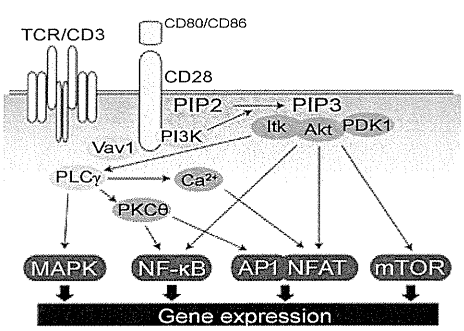

Figure 3: Costimulation signaling. Additional engagement of cell surface receptors such as CD28 provides and integrates signals required for anergy avoidance and productive T cell activation. The signaling generated by CD28 ligation has no unique effects but enhances the T cell signaling, including pathways of PI3K–Akt–mTOR, NF-κB, NFAT-AP1, and MAPK.

CD28/PI3K-generated PIP3 also provides a docking site for the PH domain of Itk. As described above, Itk associates with the TCR downstream complex consisting of LAT/SLP-76/Gads/PLCγ1 (Figure 3). Importantly, the localization and activation of this signaling complex depends on PI3K-generated PIP3.

A novel signaling mechanism has been suggested where by CD28 regulates cAMP degradation in T cell rafts through the recruitment of an Akt/β-arrestin/PDE4 complex [17]. CD28/PI3K-mediated PIP3 production recruits this supramolecular complex to lipid rafts, with recruitment occurring through the Akt PH domain.

Thus, although many of these pathways are activated by TCR ligation alone, the magnitude and maintenance of T cell activation signaling required for an appropriate response appear to be considerably regulated by costimulatory signals. This suggests that CD28 engagement primarily functions in a quantitative rather than a qualitative change in T cell activation.

CD28-mediated NF-κB pathway

Another important CD28 mediator is NF-κB signaling. CD28/PI3K generates the second messenger PIP3 that binds to various proteins harboring PH domains, including PDK1 and Akt. At present, we know that Akt is not an essential component of NF-κB signaling, at least in T cells. Akt appears to function as a rheostat; Akt can gently modulate the strength of the NF-κB cascade through its interaction with CARMA1 (Figure 3) [16]. Of note, PDK1, which is recruited to newly generated PIP3, can efficiently bind to both PKCθ and CARMA1 [18]. CD28 facilitates NF-κB activation by regulating the recruitment and phosphorylation of PDK1, which are necessary for the efficient binding of PDK1 to PKCθ and CARMA1 and thus for NF-κB induction. Furthermore, TCR/CD28 confers the noncanonical NF-κB activation in naïve T cells [19]. Taken together, NF-κB signaling is one of the major signaling pathways regulated by costimulation. Full activation of the NF-κB pathway requires CD28-mediated costimulatory signals in T cells.

CD28–Vav1 pathway

Vav1 is a guanosine exchange factor (GEF) for several small GTPases, including RAC1, RAC2, and RHOG. Vav1 controls several biochemical processes, such as those involved in cytoskeletal rearrangements. In addition, Vav1 strongly amplifies CD28-mediated costimulation-dependent activation of NFAT, NF-κB, CD28 response element, and JNK [20,21].

T cell development is markedly reduced in Vav1-deficient mice [22]. Of note, a CD28 mutant, which is unable to activate Vav1, does not alter TCR-directed ZAP-70 and LAT phosphorylation, but it does affect SLP-76, PLCγ1, and Itk phosphorylation and Akt activation [23]. This phenotype, which is remarkably similar to that of Vav1-deficient T cells [24], supports a role for Vav1 as a crucial signaling effector of CD28 costimulation.

TCR/CD28 and MAPK pathways

TCR engagement activates the ERK, JNK, and p38 cascades in T cells. With regard to costimulation effects, one study demonstrated that T cell activation involves these three MAPK cascades [25]. pMHC ligation directly activates the Ras–MEK–ERK pathway, as described above. JNK activation stimulated by TCR engagement requires CD28 coligation in T cell clones. However, JNK activation is not observed in mouse primary T cells. TCR and CD28 synergize after coligation to elicit enhanced p38 MAPK activation. p38 MAPK, but not JNK, is involved in signal integration during costimulation of naive mouse primary T cells. Indeed, the p38 MAPK inhibitor SB203580 blocks CD28-dependent proliferation and IL-2 production in human T cells [26]. We currently do not know how p38 modulates TCR signaling and IL-2 production.

Studies using both pharmacological and genetic manipulations have provided evidence that p38 is a crucial mediator of T cell development, activation, and inflammation [27]. In addition, p38α function in T and B cells has been addressed using the RAG-deficient blastocyst complementation method. Surprisingly, p38α-deficient T and B cells developed in normal numbers and proliferated normally in response to various stimuli, such as antigen–receptor ligation [28]. Moreover, mice that lack p38β appear to be completely normal with no obvious defects in T cell development or LPS-induced cytokine production [29]. To reconcile the genetic and pharmacological data with the paucity of observable abnormalities in p38 isoform-specific knockouts, further characterizations and detailed analyses of the p38 pathway in both normal T cells and T cell malignancies will be necessary.

Signaling Pathways Linking to T cell Activation, Development, and Disorder

In addition to TCR/CD28 engagements, several other signaling inputs participate in the regulation of T cell fates. These comprise pathways responsible for the development and differentiation of several cell types. Of note, deregulation of these signaling pathways lead to T cell disorders.

NF-κB signaling

In addition to TCR engagement-mediated NF-κB activation, pathological studies have strongly demonstrated that NF-κB activation is often sustained in T cell disorders. ATL cells show strong, constitutive NF-κB activation that contributes to their prolonged survival, proliferation, and invasiveness [30]. In particular, human T cell leukemia virus type 1 (HTLV-1) Tax is an intracellular stimulator of IKK. This stimulation is based on the physical interaction between Tax and IKK, which leads to the persistent activation of NF-κB-mediated transcription. Formation of the Tax/IKK complex relies on the physical interaction between Tax and the IKK regulatory subunit IKKγ. The Tax–IKKγ interaction is required for recruiting Tax to IKK catalytic subunits and for Tax-mediated IKK activation (Figure 4) [31].

In leukemic cells in which Tax is not expressed, a noncanonical NF-κB cascade appears to be important for the cellular characteristics of ATL. Both the canonical and noncanonical NF-κB pathways are persistently activated because NIK is aberrantly expressed in ATL cells [32]. NIK plays a central role in noncanonical NF-κB signaling through IKKα phosphorylation [33], and its constitutive expression leads to aberrant NF-κB activation in various malignancies, including B cell lymphoma, multiple myeloma, breast cancer, pancreatic cancer, and ATL [30].

Recently, we identified a novel molecular link between NF-κB activation and miRNA deregulation. Comprehensive gene expression analysis and *in vitro* experiments showed that miRNA-31 (miR-31) could regulate NIK expression through the 3′ untranslated region (UTR) in several cell types. In ATL cells, miR-31 expression was genetically and epigenetically silenced, which in turn induced constitutive NF-κB activation through NIK expression (Figure 4) [34]. Because current evidence clearly indicates that miR-31 dominates NF-κB activity in T cells, manipulating cellular miR-31 levels may be a novel molecular approach to reduce NF-κB activity and induce cellular apoptosis.

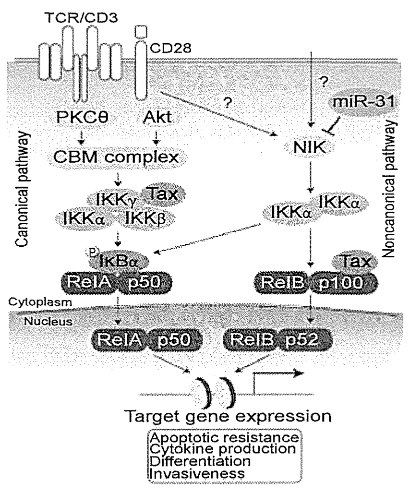

Figure 4: NF-κB signaling in T cell. Canonical and noncanonical pathways contribute to gene expression required for apoptotic resistance, inflammatory cytokine production, appropriate differentiation, and invasiveness. In HTLV-1 infected cells, viral protein Tax binds and drastically activates both pathways. In transformed leukemic cells, loss of miR-31 leads to NIK accumulation and persistent NF-κB activation.

Why is NF-κB signaling important for ATL cell survival? One of the target genes is Bcl-xl, which is expressed when NF-κB is activated. In HTLV-1-infected cell lines, Bcl-xlis expressed through the Tax–NF-κB pathway. Interestingly, fresh ATL samples exhibit Bcl-xl overexpression. Given that Bcl-xl is a principal anti-apoptotic protein as a Bcl-2 family member, persistent Bcl-xl expression is one of the molecular means to resist apoptosis, which may contribute to clinical chemoresistance. Indeed, inhibition of the NF-κB pathway by NIK depletion leads to impaired Bcl-xl expression and apoptotic death of ATL cells [34].

Because prevention of NF-κB activation showed good results in a xenograft model of cell lines with and without Tax [30], molecular targeting therapy based on the NF-κB pathway is a promising new treatment for ATL. Of note, we found that specific inhibition of NF-κB by DHMEQ could also remove virus-carrying cells from carrier peripheral blood mononuclear cell (PBMC) samples [35].

mTOR pathway

The signaling pathway target of rapamycin (TOR) is at the intersection between cell growth and starvation. The evolutionarily conserved kinase mammalian TOR (mTOR; officially known as the mechanistic target of rapamycin) controls cell growth and metabolism-related response to environmental inputs by regulating gene expression, which has been implicated in disease states, such as cancer, metabolic diseases and ageing. In T cell regulation, mTOR signaling is also involved in immune signals and metabolic cues for the proper maintenance and activation of T cells. Under resting conditions, mTOR signaling is strictly controlled by multiple inhibitory mechanisms, which enforces normal T cell homeostasis. T cell activation through antigen recognition triggers mTOR activation, which in turn influences the differentiation patterns of these cells.

mTOR, which belongs to the family of PI3K-related protein kinases (PIKK), is a conserved serine/threonine kinase and plays a central role in the regulation of cell growth and metabolism [36]. Diverse environmental signals, such as nutrients and growth factors, deliver their signaling inputs to the PI3K–Akt axis. Many upstream signals activate the mTOR complex 1 (mTORC1) via the GTP-loaded small GTPase RHEB. The activated PI3K–Akt pathway phosphorylates the Thr1462 residue of tuberous sclerosis 2 (TSC2), which in turn inhibits the TSC1/2 complex. Because RHEB is tightly regulated by the TSC1/2 complex, TCR/CD28 engagement activates RHEB/mTORC1 via the PI3K–Akt pathway. Recent studies have shown that RHEB deficiency in T cells reduced mTORC1 activation in response to TCR stimulation [37]. In addition, loss of TSC1 disrupts the TSC1/2 complex and enhances basal and TCR-induced mTORC1 activity [38,39]. Therefore, these results suggest a crucial role for the TSC–RHEB axis in T cell responses.

TCR engagement can activate both mTORC1 and mTORC2 within minutes. The strength of mTOR activation appears to be directly correlated with the duration of T cell–APC interaction and the cognate antigen dose. It should also be noted that mTOR activity is further conferred by costimulatory signals. CD28-mediated costimulation is a major driving force for the PI3K–AKT axis (described above), which in turn upregulates TCR-induced mTOR activity to facilitate productive T cell activation (Figure 5) [40,41]. In addition to CD28, another costimulatory receptor, OX40, which is a member of the tumor necrosis factor receptor (TNFR) family, assembles a signaling complex by recruiting PI3K–AKT to augment TCR-dependent Akt signaling; this may also affect mTOR signaling in T cells [42].

Several genetic studies have strongly suggested that the mTOR pathway is closely associated with T cell differentiation and homeostasis [43]. In particular, CD8$^+$ T cell differentiation is strictly regulated by mTOR signaling [44]. In addition, mTOR signaling appears to be involved in T helper (Th) cell differentiation and proliferation [43]. In sum, mTOR dictates the cell fate decisions of effector and regulatory T cells.

A common hematological malignancy, T-ALL, frequently harbors activating mutations in *NOTCH1* and/or loss-of-function mutations in a gene encoding phosphatase and tensin homolog deleted on chromosome 10 (PTEN). PTEN plays critical roles in cell growth, migration, and death because it can inhibit the PI3K–Akt pathway by catalyzing PIP3 dephosphorylation. It is noteworthy that both of these mutations can activate mTOR signaling, which suggests a pivotal role for mTOR in T-ALL development. Taken together, mTOR-mediated T cell fate decisions are of particular importance because of the unique functions of the mTOR pathway. Recent experimental advances have established that mTOR is a fundamental determinant of T cell homeostatic and functional fates. In addition, therapeutic targeting of mTOR may have beneficial effects for treating T cell disorders.

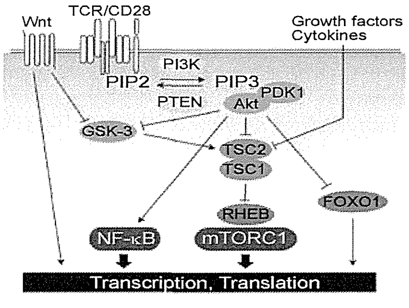

Figure 5: PI3K–Akt–mTOR axis. After TCR/CD28 stimulation, activated PI3K enhances phosphorylation of PDK1 and Akt. The Phosphorylated Akt in turn inhibits tumor suppressor TSC2, resulting in activation of mTORC1. In addition, Akt supports NF-κB signaling through CBM complex.

Notch pathway

Notch proteins are cell-surface receptors and their expression is widely conserved in numerous species. It has been shown that Notch signaling is essential for T cell lineage development, thymocyte survival, and proliferation of T cell progenitors [45]. In addition, Notch signaling has been extensively studied in T-ALL because several lines of genetic evidence highlight the Notch signaling cascade as a central player in T-ALL pathogenesis [46].

Notch proteins are synthesized as a single polypeptide (approximately 300 kDa). After intracellular modifications that include fucosylation, glysocylation, and cleavage, a Notch heterodimer associates with the plasma membrane. Signaling is triggered by the interaction of Notch with Delta-like ligands (DLLs) or Jagged ligands on the surface of instructing cells. This ligation induces proteolytic cleavage of the Notch receptor by disintegrin and metalloproteinase (ADAM) and mono-ubiquitination of the intracellular portion of the transmembrane Notch fragment. This final cleavage leads to the release of the Notch intracellular domain from the plasma membrane and its translocation to the nucleus.

CBF1/suppressor of hairless/Lag1 (CSL) represses transcription of Notch target genes. Following activation by Notch, CSL is converted into a transcriptional activator and activates the transcription of the same genes. Intracellular Notch displaces the corepressors from CSL and recruits coactivators, such as Mastermind-like 1 (MAML1), to activate the expression of target genes (Figure 6). Intracellular Notch is then degraded by a polyubiquitination–proteasome pathway.

A landmark study published in 2004 changed our perspectives dramatically by showing that more than 50% of T-ALL cases harbored Notch1 mutations, which resulted in the hyperactivation of the Notch pathway. This finding implicated Notch1 has a very important role in the pathogenesis of T-ALL [47]. This suggests that Notch likely interacts with several important cellular pathways and can cooperate with other oncogenes during leukemogenesis (discussed above).

Although the importance of Notch signaling activation in T-ALL is well established, the detailed molecular mechanisms by which NOTCH1 induces T cell transformation remain unclear. The best-characterized direct target genes are the bHLH transcriptional repressor Hes1 and the transcription factor c-Myc. Recently, Hes1 was shown to be a key regulator in the induction and maintenance of T-ALL. Notch-mediated c-Myc expression is also crucial for maintaining T-ALL.

Recently, activating mutations in Notch were identified in more than 30% of ATL patients [48]. Of note, compared with the activating frameshift mutations observed in T-ALL, ATL showed single substitution mutations activating the Notch pathway. These gene mutations could reduce CDC4/Fbw7-mediated degradation, which resulted in stabilization of the intracellular NOTCH1. They also resulted in Hes1 expression in ATL patient samples. In addition, γ-secretase inhibitors reduced tumor cell proliferation and tumor formation in ATL-engrafted mice. Collectively, these findings suggest that activated Notch may be important for ATL leukemogenesis.

There is mounting evidence that Notch signaling is also an important pathway for mediating cell fate decisions in developing thymocytes and peripheral T cells. Strong Notch signaling restricts the multilymphoid progenitors of the T cell lineage and promotes thymocyte development [49]. Results from several studies support a role for Notch in the generation of single-positive cells from double-positive precursor thymocytes [50].

Notch proteins are now emerging as potentiators of TCR/CD28 signaling in mature T cells. Some studies have confirmed the roles for Notch receptors in T cell activation, proliferation, and cytokine production. Pharmacological inhibition of the Notch pathway in $CD4^+$ and $CD8^+$ T cells significantly inhibited their proliferation and IFNγ production after TCR stimulation [51]. In addition, Notch signaling was shown to be involved in a positive-feedback loop of IL2–IL2R that regulated T cell proliferation [52]. Important pathways activated through Notch1 signaling include the PI3K–Akt and mTOR signaling cascades. In addition, NF-κB signaling, which can be activated through TCR/CD28 signaling, appears to be sustained by the Notch pathway. These details are discussed in a later chapter.

Wnt pathway

Wnt signaling is subject to strict molecular control. Thus, dysregulated Wnt signaling has been implicated in the development of several malignancies, including the leukemias and lymphomas [53]. To date, at least three different Wnt pathways have been identified: the canonical Wnt pathway, which involves β-catenin and the T cell factor (TCF)/lymphocyte-enhancer-binding factor (LEF) family; the planar cell polarity pathway; and the Wnt/Ca^{2+} pathway. Among these, the canonical (Wnt/β-catenin) pathway is primarily involved in T cell development and proliferation [54].

One important study demonstrated that a T cell-specific defect in Wnt signaling by deletion of β-catenin impaired T cell development at the β-selection checkpoint, which resulted in a decrease in splenic T cells. In addition, β-catenin appeared to be a target of TCR signals in thymocytes and mature T cells. These results indicate that β-catenin-mediated signals are required for normal T cell development [55].

TCF1 is highly expressed in T cells. A recent study showed that β-catenin expression was stabilized after TCR-mediated T cell stimulation, resulting in the upregulation of TCF1-dependent gene expression in T cells [56]. It also suggested that TCF-1 initiated Th2 differentiation of activated $CD4^+$ T cells by promoting GATA-3 expression and suppressing IFN-γ expression. In addition, during T cell development, TCR signaling was shown to stabilize β-catenin and regulate the response to αβTCR engagement [57]. In sum, several genetic studies have suggested that the canonical Wnt pathway participates in T cell development. In $CD8^+$ T cells, activation of Wnt/

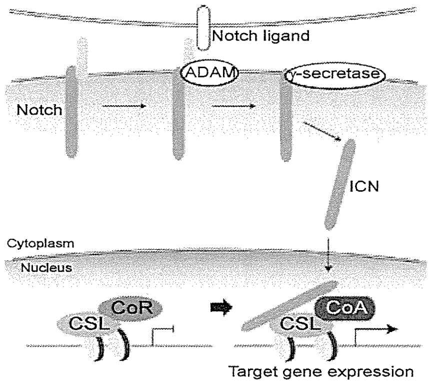

Figure 6: Notch signaling. After intracellular modifications, the Notch heterodimer associates with the plasma membrane. Signaling is triggered by the interaction with Notch ligands, which induces proteolytic cleavage of the Notch and leads to the nuclear translocation of the Notch intracellular domain (ICN). The intracellular Notch displaces the co-repressors (CoR) from CSL and recruits co-activators (CoA) to activate the expression of target genes.

β-catenin signaling inhibited effector differentiation and promoted the generation of self-renewing CD8$^+$ memory stem cells and central memory T cells. In CD4$^+$ T cells, Wnt/β-catenin signaling facilitated Th2 polarization and enhanced the survival of naturally occurring regulatory T cells (nTreg). Canonical Wnt signaling has also been suggested as important for the self-renewal of hematopoietic stem cells (HSCs) and progenitor compartments. β-catenin deletion perturbs HSC survival, self-renewal, and their subsequent development into T cells [58,59].

Because the Wnt pathway is important for normal T cell development and signaling pathways, constitutively active Wnt signals can lead to T cell acute malignancies. An experimental model of Wnt activation demonstrated that the constitutively active form of β-catenin led to development of an aggressive T cell lymphoma that could invade the bone marrow and was transplantable into irradiated recipient mice [60]. These data suggest that Wnt-dependent c-Myc expression is required for lymphomagenesis. Interestingly, this appears to be independent of Notch signaling that has been thought to be an analog of the Wnt pathway. Crosstalk between these pathways will be discussed in a later chapter.

Interestingly, modest transgenic expression of β-catenin in thymocytes enhances the generation of CD8$^+$ thymocytes and accelerates thymic involution [61]. Some evidence indicated that oncogenic β-catenin induced p53-independent oncogene-induced senescence and p53-dependent apoptosis, which protected developing thymocytes from transformation [62]. The molecular mechanisms induced by the Wnt pathway in T cells remain to be elucidated.

Hedgehog (Hh) pathway

The Hh family of secreted intercellular molecules, including Sonic, Indian, and Desert Hh, regulates target gene transcription through GLI transcription factors. This family is of pivotal importance in embryogenesis and the homeostasis of adult tissues, as well as cell differentiation, cell cycle progression, and cell survival [63].

Hh signaling is important in T cell development in both humans and mice [64].The generation of mature functional T cells in the thymus requires sonic Hh (Shh) signals from the thymic epithelium. Shh-induced signaling is important for the differentiation and proliferation of early thymocyte progenitors. Shh also modulates TCR signaling during repertoire selection.

Hh signaling is involved in the conversion of immature DP cells to mature SP thymocytes and can affect TCR repertoire selection and CD4/8 lineage choices. Depletion of Hh signaling promotes CD4 lineage commitment and results in an increased CD4:CD8 ratio. In contrast, increased Hh signaling results in a reduced CD4:CD8 ratio, suggesting that Hh signaling is an inducer of CD8$^+$ T cell development [64,65].

Hh is also involved in peripheral T cell activation. Additional Shh supports TCR/CD28 signaling and enhances T cell activation and subsequent proliferation [66-68]. In contrast, Hh activation by the expression of the constitutive active form of Gli2 results in inhibition of TCR/CD28-mediated T cell activation. In this case, Hh appears to inhibit TCR-dependent Erk phosphorylation [65]. Modulation of TCR signal strength by Hh has profound implications for immunity. Thus, the precise molecular involvement of Hh in T cell signaling needs to be examined.

Hh signaling is implicated in several hematological malignancies, including chronic lymphocytic leukemia (CLL), plasma cell myeloma, mantle cell lymphoma, diffuse large B-cell lymphoma (DLBCL), ALK-positive anaplastic large cell lymphoma (ALCL), chronic myelogenous leukemia (CML), and acute leukemias (AML). ALK+ ALCL is an aggressive type of non-Hodgkin's lymphoma of the T cell/null lineage. One study demonstrated that Hh ligands and GLI1 were highly expressed in ALK+ ALCL [69]. NPM-ALK activates the PI3K–Akt pathway, which contributes to GSK-3β inactivation. Because GLI1 and GLI2 are the targets of GSK-3-mediated proteasomal degradation, NPM-ALK activates Hh signaling through the PI3K–Akt–GSK-3 cascade. Of note, we have demonstrated that the NPM-ALK abrogates CD30 signaling and constitutive NF-κB activation in ALCL cells [70]. The reciprocal relationship between Hh and NF-κB signaling in T cells remains unclear.

TGF-β-mediated signaling

The TGF-β superfamily regulates many cellular functions, including cell growth, differentiation, migration, and apoptosis. TGF-β-mediated signaling is essential for embryonic development, embryogenesis, and cell fate decisions [71].

TGF-β is synthesized in an inactive form consisting of a TGF-β dimer in association with the latency-associated protein (LAP). Signaling is initiated by a conformational change in a tetrameric complex consisting of TGF-β receptor II (TGF-βRII), TGF-β receptor I (TGF-βRI), and activated TGF-β dimer. Activated TGF-βRI then phosphorylates the transcription factors Smad2 and Smad3 and triggers their translocation into the nucleus. Smads then bind to the specific regulatory sequences of target genes and regulate gene expression by recruiting transcription cofactors.

TGF-β regulates T cell development, homeostasis, tolerance, and immunity [72]. TGF-β signaling promotes the differentiation of thymic T cells into natural killer T cells, nTreg cells, and CD8$^+$ T cells. TGF-β signaling is likely to be pivotal for the survival of peripheral CD4$^+$ and CD8$^+$ T cells with low affinity. In contrast, it inhibits the proliferation and differentiation of CD4$^+$ and CD8$^+$ T cells with high affinity.

In particular, TGF-β plays central roles in Treg cell regulation. It inhibits proliferation of Treg cells, although it supports their maintenance in peripheral lymphoid organs, which can control Treg cell survival. TGF-β promotes the differentiation of induced Treg (iTreg) cells, which is potentiated by IL-2 and retinoic acid (RA). In the presence of IL-6, TGF-β drives the differentiation of Th17 cells and supports their maintenance.

The interplay between TCR and TGF-β signaling has been implicated in the regulation of several genes, including FoxP3, which is important for Treg functions [73]. However, because most studies have mainly performed phenotypic analyses, the exact molecular signaling pathway induced by TGF-β in T cells is not completely understood.

Signalings from receptors of innate immune cells

Innate and adaptive immune responses have many interactions that are regulated by the balance of signals initiated by several activatory and inhibitory receptors. Toll like receptor (TLR) expression has been generally assigned to professional APCs, such as dendritic cells. However, several recent studies have demonstrated that the low expression levels of TLRs could also be detected on naïve and memory T cells [74,75]. In addition, TLR agonists can directly target TLRs expressed on T cells and function as signal inducers.

Engagement of the TLR1/2 complex was associated with T cell proliferation, survival, and functions [76]. Ligation of TLR3 on effector CD8$^+$ T cells increased IFN-γ secretion by these cells. Interestingly, the effects of CpG (TLR9 ligand) on CD4$^+$ T cells were mediated by MyD88 and PI3K-dependent mechanisms [77]. Another study showed that ligands for TLR3 and TLR9 directly enhanced T cell survival, which was sustained by NF-κB signaling [78]. Collectively, these findings suggest that TLR agonists can directly target TLR signaling in effector CD4$^+$ and CD8$^+$ T cells. We do not understand the molecular mechanisms by which TLR signaling can affect the other sustained T cell signaling.

NKG2 receptors are expressed by natural killer (NK) cells and a subset of CD8$^+$ T cells. They regulate the development and function of NK cells by inhibiting and activating cytotoxicity and promoting cell survival. The CD94/NKG2 heterodimers consist of one CD94 molecule and one NKG2 family member, including NKG2A and its splice variant NKG2B, which form inhibitory receptors, as well as NKG2C, NKG2E, and NKGH, which form activating receptors [79]. NKG2D is unique in that it does not dimerize with CD94; instead, it associates with the adaptor molecules DAP10 and DAP12 for its activating function [80]. Among these, the acquisition of CD94/NKG2A expression by CD8$^+$ T cells could play a role during infection by pathogens. This has been observed in mice infected with a variety of pathogens, including lymphocytic choriomeningitis virus, herpes simplex virus (HSV)-1, influenza virus, polyomavirus, and *Listeria monocytogenes* [81]. Furthermore, HIV-1 infection has also been implicated in the upregulation of CD94/NKG2A [82,83]. The expression of CD94/NKG2A, which is acquired early during the infection and persists after clearance of the pathogen, does not interfere with the activity of CD8$^+$ T cells. However, the CD94/NKG2A expression inhibits antiviral CD8$^+$ T cell responses [84]. In addition, high level of CD94/NKG2A expression on CD8$^+$ T cells protects them from apoptosis, thereby perhaps inducing in the generation and maintenance of memory cells.

Emerging Regulatory Factors: Learning from Malignancies

Recent experimental evidence indicates the presence of additional molecular hallmarks of T cells. miRNAs regulate gene expression patterns in all cell types. Epigenetic gene regulation is based on chromatin regulation. Both of these are pivotal for development, differentiation, proliferation, and other cellular processes in hematopoietic lineage. Their deregulation is frequently observed in T cell disorders, which strengthens the fact that miRNA-mediated gene regulation and epigenetic mechanisms may be important for maintaining T cell homeostasis.

miRNA in T cells

One of the most significant recent advances in biomedical research has been the discovery of the 22-nt-long class of non-coding RNA designated miRNA that post-transcriptionally regulates gene expression by binding to the target mRNAs. miRNA is expressed by all metazoans and plants, as well as by several DNA viruses; it regulates cellular processes such as development, differentiation, growth, homeostasis, stress responses, apoptosis, and immune activation [85].

Some recent extreme studies have collectively suggested several concepts. First, miRNAs have unique roles as time-sensitive gene regulators. Second, miRNAs have distinct functions in distinct cell types. Third, miRNAs regulate gene expression by incompletely repressing their targets. These specific functions of miRNAs as "fine-tuners" are apparently involved in immune systems as well as hematological cancers. It is not surprising that miRNA deregulation appears to play important roles in human cancers.

miRNAs provide modest changes in gene expression [86]. However, it is also true that gene expression patterns can occasionally be changed drastically by a change in a single miRNA that regulates a key transcription factor. For example, a decrease in a miRNA, miR-150, leads to drastic deregulation of the B cell transcriptome by regulating c-MYB [87]. Furthermore, given that miRNAs are upstream of gene regulation systems, miRNAs confer robustness to several biological processes by controlling several feed-forward and feed-back loops [86]. Several recent reports including our own have demonstrated crosstalk between miRNA-mediated gene regulation and several signal transduction pathways in T cells.

Specific knockout of *Dicer*, which is essential for processing miRNAs and siRNAs in the T cell lineage, leads to impaired T cell development and aberrant Th cell differentiation and cytokine production [88,89]. In addition, peripheral CD8$^+$ T cell development was severely blocked after *Dicer1* deletion in the thymus. These cells were defective in miRNA processing, and after stimulation, they proliferated poorly, which resulted in their apoptosis. Dicer-deficient Th cells preferentially expressed INFγ. The levels of secreted IL-2 in *Dicer*-deficient and control cultures were comparable after primary stimulation, and addition of exogenous IL-2 to the growth media did not rescue the proliferative defect of *Dicer*-deficient T cells, which suggested that Dicer might be involved in IL-2 receptor (CD25) signaling in T cells [89].

An early study revealed that miR-181a acted as an intrinsic antigen sensitivity "rheostat" during T cell development [90]. miR-181a expression enhanced the basal activation of TCR signaling and IL-2 production because miR-181a repressed multiple negative regulators of TCR signaling (Figure 7). Thus, several miRNAs probably participate in each step of T cell regulation. This in turn suggests that miRNA deregulation may cause catastrophic gene regulation in T cells.

Individual miRNAs have been implicated in T-ALL. miR-19b is

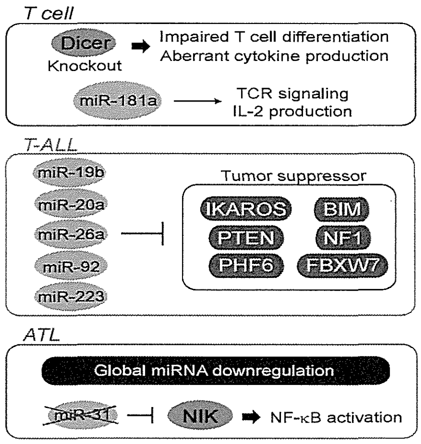

Figure 7: miRNA in T cell biology. Specific knockout of *Dicer* in T cell lineage leads to impaired T cell development and aberrant T helper cell differentiation and cytokine production. miR-181a expression enhances the basal activation of TCR signaling and IL-2 production (top box). In T-ALL cells, upregulation of five miRNA leads to repression of tumor suppressor genes implicated in T-ALL (middle box). Global downregulation of miRNA is the one of molecular hallmarks of ATL. In particular, miR-31 expression is genetically and epigenetically silenced, which is prerequisite condition of ATL cells. The loss of miR-31 consequently contributes to NIK over expression.

a member of the oncogenic miR-17-92 cluster, which is targeted by the t(13;14)(q32;q11) translocation in T-ALL [91]. Comprehensive methods have been used to address the miRNA candidates associated with abnormal gene expression and leukemogenesis. Recently, one study reported on miRNA signature in T-ALL [92]. A total of 430 types of miRNAs were analyzed in 50 clinical T-ALL and 18 T-ALL cell lines by quantitative RT-PCR, revealing that a small set of miRNAs was responsible for the cooperative suppression of several tumor suppressor genes. Cross comparisons of miRNA expression profiles in human T-ALL with the results of an unbiased miRNA library screen identified five miRNAs (miR-19b, miR-20a, miR-26a, miR-92, and miR-223), which were capable of promoting T-ALL development in a mouse model. Furthermore, these miRNAs produced overlapping and cooperative effects on tumor suppressor genes implicated in T-ALL, including *IKAROS, PTEN, BIM, PHF6, NF1,* and *FBXW7.* Thus, a comprehensive and unbiased analysis of miRNA actions in T-ALL reveals a striking pattern of miRNA–tumor suppressor gene interactions (Figure 7).

For ATL, some studies have reported several miRNA aberrations identified in HTLV-1-infected cells and ATL samples [93-95]. Very recently, we established global gene expression analysis in a large cohort ATL study that included analyses of mRNA expression, miRNA levels, and genomic copy numbers [34]. A strict threshold ($p < 1 \times 10^{-5}$) and two-dimensional hierarchical clustering analysis revealed 61 miRNAs with significantly altered expression levels in ATL cells ($n = 40$) compared with control $CD4^+$ T cells ($n = 22$). It is most important that primary ATL samples show global miRNA downregulation, similar to observations in other cancer researches [96,97]. Of the 61 miRNAs, 59 (96.7%) exhibited decreased expression in ATL (Figure 7).

Among these, miR-31 was the most profoundly repressed miRNA in all ATL individuals (fold change of 0.00403). This is a known tumor suppressor that may also be associated with metastatic breast cancer [98]. Other downregulated miRNAs found in ATL patients may also be involved in the hallmark capabilities of ATL because they were uniformly decreased in tested ATL samples and each miRNA may regulate a large number of genes.

Several predictions and experimental approaches have defined a novel miR-31 target gene, NIK, which acts as a persistent NF-κB activator in various malignancies (described above). Manipulation of the miR-31 level clearly indicated that the miR-31 level was negatively correlated with cellular NF-κB activity. Importantly, enforced miR-31 expression in B cells attenuated both BAFF- and CD40L-mediated NIK accumulation and subsequent canonical and noncanonical NF-κB signaling. Induced miR-31 expression or NIK knockdown reduces apoptotic resistant proteins such as BCL-XL and XIAP, which results in significant apoptosis among ATL cell lines as well as among primary leukemic cells from ATL patients [34]. Several lines of evidence definitively support two notions: (1) miR-31 acts as a tumor suppressor in T cells and (2) NIK-regulated NF-κB is of pivotal importance to cancer cell survival (Figure 7).Taken together, based on the results of genetic studies and those on T-ALL and ATL, abnormal miRNA expression has a critical impact on the development of hematological malignancies.

Epigenetic regulation

Technological advances in genomics and epigenomics have provided new methods to distinguish one cell type from another. The epigenetic code consists of the combined on–off states of hundreds of genes, which coordinately dictate cellular identity and function. Increasing attention is being paid to global regulatory factors and molecular mechanisms by which gene transcription control is regulated. Within these environments, genome programming operates fundamentally through DNA methylation, histone chemical modification, and protein complex binding.

The appropriate control of gene expression must be maintained in normal T cells by utilizing transcriptional networks as well as epigenetic controls. Indeed, several specific, comprehensive studies have demonstrated the epigenetic regulation of genes encoding for cytokines that are required for T cell development, differentiation, and activation [99]. Collectively, several global analyses have proposed that naïve T cell activation, differentiation, and lineage commitment result in epigenetic changes and that a fine balance between different histone modifications is required. In contrast, memory T cells are already poised and do not require epigenetic changes for short-term activation. It has been suggested that transcriptional memory, particularly epigenetic marks on chromatin, forms the underpinning of immunological memory [100-102].

Integrated histone modifications consequently decide the degrees of chromatin condensation and subsequent transcriptional sensitivity. Trimethylation of the histone H3 Lys27 (H3K27) mark plays a central role in repressing transcription, primarily in the euchromatin region. The polycomb family is a master regulator of the H3K27me3 level by inducing and maintaining the histone mark. Polycomb repressive complex 1 (PRC1) and PRC2 are recognized as essential molecular machines involved in polycomb-mediated gene silencing [103].

The polycomb family participates in the control of T cell activation. Enhancer of zeste homolog 2 (EZH2) serves as the catalytic subunit in PRC2 and mediates gene silencing by catalyzing H3K27 trimethylation in the promoters of target genes. One study showed that triggering of TCR and costimulation signaling in *Ezh2*-decificint T cells resulted in an impaired proliferative response [104]. The mechanism by which Ezh2 can affect T cell signaling is not completely understood.

Deregulation by the polycomb family confers a specific gene expression pattern that is responsible for chronic proliferation, survival, peculiar development, and cancer-associated stemness in various cancer types, including ATL [105]. The involvement of the polycomb family in ATL was first revealed by global gene expression analysis. Significantly higher levels of EZH2 as well as RING1- and YY1-binding protein (RYBP) transcripts with enhanced H3K27me3 levels were found in ATL cells compared with normal $CD4^+$ T cells [106]. EZH2 is highly expressed in many cancer types, including breast and prostate cancers and lymphomas, and its expression is often correlated with advanced stages of tumor progression and a poor prognosis. We recently identified a notable gene silenced by polycomb. A human gene that encodes for miR-31, *hsa-miR-31*, is located at 9p21.3, which is adjacent to clusters of the *CDKN2* and *IFNA* families. In addition to loss of this gene (12.5% of ATL cases), transcription of the miR-31 precursor was completely lost in ATL cells. Computational predictions and experimental evidence clearly demonstrated that an assembly of YY1-binding motifs upstream of the miR-31 region was responsible for the occupancy of the polycomb family at the target region, which resulted in H3K27me3-dependent transcriptional repression. Of note, given that miR-31 is a master regulator of the ATL-specific gene expression pattern described above, polycomb-mediated loss can influence gene expression downstream of miR-31in T cells and other cell types (Figure 8) [34].

Intriguingly, several recent lines of evidence have shown two faces