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RESEARCH ARTICLE

Correlation of Wnt and NOTCH pathways in esophageal squamous cell carcinoma

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Abstract There is an inevitable association between cell signaling pathways and tumorigenesis. Wnt and notch pathways play important roles during development and self-renewal. Beside the independent role of such pathways on tumor progression, different cross talks between these pathways through tumorigenesis are emphasized. In this study, we analyzed cross talk between Wnt and NOTCH signaling pathways through assessment of probable correlation between MAML1 and PYGO2 as the main transcription factors of these pathways, respectively in esophageal squamous cell carcinoma (ESCC) patients. Levels of MAML1 and PYGO2 mRNA expression in 48 ESCC patients were compared to the correlated margin normal tissues using real-time polymerase chain reaction (PCR). Eleven out of 48 patients (22.9 %) have shown the concomitant MAML1/PYGO2 over expression in significant correlation with tumor size ($p = 0.046$) and depth of tumor invasion ($p = 0.050$). We showed that there is a significant correlation and feedback between these markers during the ESCC progression and metastasis.

Keywords Cancer stem cell · Signaling pathway · Crosstalk · Feedback · Gene expression · Real time PCR

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Introduction

Northeastern Iran is one the most prevalent areas for esophageal squamous cell carcinoma (ESCC), (Gholamin et al. 2009). Despite new advances in prognosis, diagnosis, and therapeutic methods, majority of patients are diagnosed usually in advanced pathological tumor stage (III/IV) having extended metastasis of tumor cell throughout the body which prevents an efficient surgery (Headrick et al. 2002; Hulscher et al. 2002). Regarding the importance of early diagnosis in patients' survival, elucidation of molecular mechanisms involved in ESCC progression and metastasis is essential to introduce new prognostic and therapeutic targets.

Developmental cell signaling pathways are key players of both embryonic development and tumorigenesis. Among these different pathways, Wnt and NOTCH signaling cascades are truly involved in cancer progression. Wnt signaling pathway is activated via the binding of secreted Wnt ligands to the cysteine-rich domain of Frizzled (Fzd) family receptors. Subsequently, signal transduction continues through 'canonical' or 'non-canonical' pathways. In Canonical pathway, Wnt/Fzd binding activates Dishevelled (Dvl), which prevents the β-catenin phosphorylation and ubiquitination, leading to β-catenin stabilization and its cytoplasmic accumulation. Then β -catenin enters to the nucleus, where it binds to the transcriptional complex comprising the TCF-1, BCL-9, and Pygopus (PYGO2) (Klaus and Birchmeier 2008). PYGO2 is one of the essential components in canonical Wnt/β-catenin

transcriptional complex (Belenkaya et al. 2002) which is involved also in tumorigenesis (Popadiuk et al. 2006) and development (Song et al. 2007). PYGO2 has a C-terminal plant homeo-domain (PHD) which can bind to the histone methylated lysines (H3K4me) and activate transcription through the recruitment of β-catenin/BCL9 on the methylated histones in promoter sequences of Wnt target genes (Fiedler et al. 2008; Gu et al. 2009). It has been shown that malfunction of canonical pathway is associated with tumorigenesis (Fukui et al. 2005; Winn et al. 2005; You et al. 2004). PYGO2 directly or indirectly regulates critical cell cycle genes such as *cyclin D1* and *p21*, leading cells to G1–S phase transition (Gu et al. 2009). Moreover, it has been shown that the PYGO2 expression in HeLa cells is involved in anti-apoptotic activity through DNA fragmentation, *caspase-9/3* activation, and indirectly blocking of BCL-2 as an anti-apoptotic factor (De et al. 2009). In Wnt pathway the PYGO2 functions as a mediator between the chromatin remodeling complex and transcriptional machinery through its evolutionarily conserved PHD domain (Kessler et al. 2009).

NOTCH signaling is a cell–cell contact dependent pathway which influences cell fate decision through a family of four transmembrane receptors including Notch1–Notch4 (Luo et al. 2005). These receptors are activated by cell surface ligands of neighboring cells. After activation, the Notch intracellular domain (NICD) releases into the cytoplasm via a proteolytic process. Subsequently, the NICD enters to the nucleus and activates CSL (CBF/RBP-Jk, suppressor of Hairless, LAG-1) transcription factors which are the main factors of the NOTCH signaling transcription machinery. Homologous mammalian mastermind- like (MAML) proteins, especially MAML1, are also included in this transcriptional machinery as coactivators (Lin et al. 2002; Wu et al. 2002). In the absence of NICD, CSL suppresses the transcription of NOTCH target genes through binding to the regulatory cis-acting elements of promoter and recruitment of SMRT (silencing mediator of retinoid and thyroid receptors) co-repressors (Kao et al. 1998; Oswald et al. 2002). Furthermore, it has been shown that MAML1 is associated with a variety of key proteins such as p53, β-catenin, and NF-kB (Jin et al. 2010; Zhao et al. 2007). MAML1 recruits different co regulators, such as histone acetyl transferase (HAT) p300, which acetylates histone H3 and H4 tails in chromatin leading to formation of an active transcriptional region (Saint Just Ribeiro et al. 2007).

Considering the importance of different cell signaling pathways in ESCC progression and development (Moghbeli et al. 2013a, b, 2014), in the present study we assessed the probable correlation between the NOTCH and Wnt signaling pathways in ESCC samples. Although there are several reports about the Wnt/NOTCH associations in different cancers, there is not any report describing their association in ESCC. Therefore, to investigate the probable involvement of Wnt/NOTCH associations in ESCC tumorigenesis, we compared the levels of *MAML1* and *PYGO2* mRNA expressions in tumor with corresponding normal esophageal tissues and evaluated their probable correlations with the clinicopathological features of the patients.

Materials and methods

Tissue samples

Forty eight ESCC patients who were undergone the tumor resection were gathered from Omid, Qaem, and Imamreza Hospitals of Mashhad University of Medical Sciences (MUMS). All the cases have not received any chemo radiotherapeutic treatments before the surgery and the tumor specimens involved at least 70 % of tumor cells. Fresh tissues (tumor and margin normal) were preserved in RNA later solution (Qiagen, Hilden, Germany) and stored at −20 °C before the mRNA extraction. The study was approved by ethic committee of Mashhad University of Medical Sciences and all patients declared their informed consent.

RNA extraction, cDNA synthesis, comparative RT-PCR and statistical analysis

Total RNA extraction and cDNA synthesis were performed as described before (Moghbeli et al. 2013a, b, 2014). Levels of MAML1/PYGO2 mRNA expression were evaluated in duplicate reactions via the comparative relative RT-PCR (SYBR Green method, GENETBIO, Korea/Stratagene Mx-3000P, La Jolla, CA) (Forghanifard et al. 2012; Moghbeli et al. 2013a, b, 2014). Correlation between the levels of MAML1/PYGO2 mRNA expression and continuous and qualitative clinicopathological features of tumors were assessed by the Pearson's/Spearman and ANOVA/t-tests, respectively (significant p value of <0.05, SPSS 16.0, Chicago, IL).

Results

Study population

Sample selection was restricted based on specific criteria in which the samples should not receive any chemo-radio therapeutic modalities prior the tumor resection and at least 70 % of tumor tissue should be comprised of tumor cells. Therefore the cases that were deprived of such prerequisites were excluded and 48 cases were finally enrolled in the present study. Age of patients at the time of diagnosis was ranged from 30 to 83 years (mean ± SD: 61.85 ± 12.33 years). Tumor size was ranged between 1.5 and 12 cm (mean ± SD: 4.23 ± 1.91 cm). All the Clinicopathological features of patients are summarized in Table 1.

Table 1 Correlation between level of MAML1/PYGO2 mRNA expression and clinicopathological features of ESCC patients

	Total	PYGO2 overexpression	MAML1 overexpression	PYGO2/ MAML1 overexpression	*P Value*
Patients	48	17(35.4 %)	21(43.8 %)	11(22.9 %)	
Mean age (mean ± SD)	61.85 ± 12.33	63.41 ± 2.49	62.05 ± 2.16	61.55 ± 3.47	
Size (mean ± SD)	4.23 ± 1.91	3.49 ± 0.40	3.79 ± 0.32	3.55 ± 0.54	
Sex					0.270
Male	28(58.3 %)	11(64.7 %)	15(71.4 %)	8(72.7 %)	
Female	20(41.7 %)	6(35.3)	6(28.6 %)	3(27.3 %)	
Location					0.411
Lower	21(43.8 %)	9(52.9)	8(38.1 %)	6(54.5 %)	
Middle	27(56.2 %)	8(47.1)	13(61.9 %)	5(45.5 %)	
Grade					0.433
P.D	8(16.7 %)	4(23.5 %)	5(23.8 %)	3(27.3 %)	
M.D	31(64.6 %)	10(58.8 %)	12(57.1 %)	7(63.6 %)	
W.D	9(18.8 %)	3(17.6 %)	4(19.0 %)	1(9.1 %)	
Lymph node					0.473
Yes	22(45.8 %)	7(41.2 %)	9(42.9 %)	4(36.4 %)	
No	26(54.2 %)	10(58.8 %)	12(57.1 %)	7(63.6 %)	
Stage					0.804
I/II	29(60.4 %)	10(58.8 %)	14(66.7 %)	7(63.6 %)	
III/IV	19(39.6 %)	7(41.2 %)	7(33.3 %)	4(36.4 %)	
Depth of tumor invasion (T)					**0.050**
T1	1(2.1 %)	1(5.9 %)	1(4.8 %)	1(9.1 %)	
T2	7(14.6 %)	2(11.8 %)	2(9.5 %)	–	
T3	40(83.3 %)	14(85.4 %)	18(85.7 %)	10(90.9 %)	
Age					0.428
<60	17(35.4 %)	6(35.3 %)	8(38.1 %)	5(45.5 %)	
≥60	31(64.6 %)	11(64.7 %)	13(61.9 %)	6(54.5 %)	
Size					**0.046**
<3	8(16.7 %)	6(35.3 %)	4(19.0 %)	4(36.4 %)	
≥3	40(83.3 %)	11(64.7 %)	17(81.0 %)	7(63.6 %)	

Bold values indicate significant correlation between mRNA expression and clinicopathological feature

Levels of *MAML1*/*PYGO2* mRNA expression in ESCC patients

Levels of *MAML1*/*PYGO2* mRNA expressions were assessed through the comparative relative real time PCR in tumor specimens in comparison with their corresponding margin normal tissues. Seventeen out of 48 cases (35.4 %) and 21 out of 48 samples (43.8 %) showed *PYGO2* and *MAML1* over expression, respectively. Concomitant overexpression of *Maml1*/*Pygo2* was detected in eleven out of 48 patients (22.9 %). Over and under-expression of the genes were defined with more than +2 fold and less than −2 fold in level of mRNA expression, respectively. The normal expression was considered for the cases between these two thresholds. The minimum and maximum fold changes of gene expression were −4.70 and 12.30 (mean ± SD: 1.66 ± 3.23) and −3.31 and 15.10 (mean ± SD: 1.27 ± 2.86) for the *MAML1* and *PYGO2*, respectively (Fig. 1).

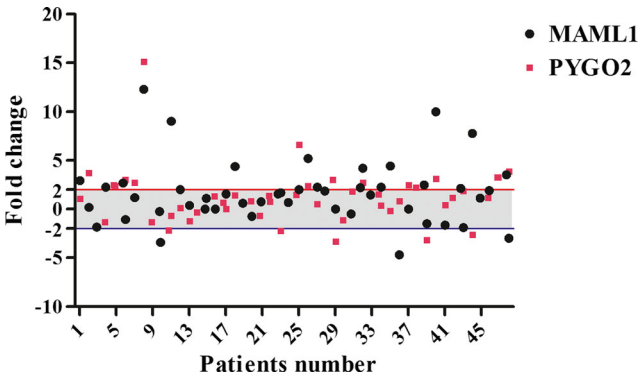

Fig. 1 Scatter plot represent descriptive analysis of relative gene expression of MAML1 and PYGO2 in ESCC patients. The thresholds for the over and under expressed cases are shown by the *red* and *blue lines*, respectively. The *grey area* mentions to the cases with normal levels of MAML1 and PYGO2 mRNA expression

Clinicopathological features and PYGO2/MAML1 mRNA expression

Previously we have reported the clinicophatological relevance of *Pygo2* and *Maml1* mRNA expression in ESCC patients in separate reports (Forghanifard et al. 2012; Moghbeli et al. 2013a, b, 2014). In the present study we assessed their concomitant expression in ESCC samples and evaluated their correlations with clinicopathological features. Overexpression of both genes was detected in eleven out of 48 (22.9 %) ESCC tissues. There was a significant correlation between the *Pygo2*/*Maml1* overexpression and depth of tumor cell invasion especially in invaded tumor cells to the adventitia (T3, 10/11, 90.9 %) ($p = 0.040$). There was not any significant correlation between the concomitant overexpression of *Pygo2*/*Maml1* and the other clinicopathological features of patients such as grade, tumor stage, lymph node status and location. Having analyzed the samples with concomitant overexpression of the genes, we found that most cases were observed among the male patients 8/11(72.7 %). Furthermore, the majority of these tumor samples were moderately differentiated (7/11, 63.6 %) in advanced stages of tumor progression (III/IV, 7/11, 63.6 %). In addition, these tumors were distributed in lower and middle parts of esophagus with 6/11(54.5 %) and 5/11(45.5 %), respectively. Moreover, the minority of such cases showed lymph node metastasis (4 out of 11, 36.5 %). Although there were not any significant correlation between PYGO2/MAML1 overexpression and continuous clinicopathological features such as age and tumor size, the *Pygo2*/*Maml1* over expressed patients had noticeably lower ages in comparison with the only *Maml1* and *Pygo2* up regulated cases (62.60 ± 2.67 vs. 66.83 ± 2.96 years old, respectively). Patients with ≤60 years old had higher levels of *Pygo2* and *Maml1* mRNA expression in comparison with others who were 60 years old (mean ± SD of fold changes: 2.14 ± 0.94 vs. 0.79 ± 0.36 and 2.44 ± 0.90 vs. 1.23 ± 0.52, respectively). In the case of tumor sizes, it seems that overexpression of these genes is inversely correlated to the size of tumors. Indeed, the tumors without any overexpression of the genes were interestingly bigger in size compared to the *Pygo2*/*Maml1* overexpressed tumors (mean ± SD: 4.90 ± 0.49 cm vs. 3.55 ± 0.54 cm). There was a significant correlation between tumor size and *Pygo2*/*Maml1* mRNA expression in which 19 out of 21 (90.5 %) cases without any overexpression in these markers were bigger than 3 cm ($p = 0.046$). The *Maml1* over expressed tumors were also bigger than the *Pygo2* up regulated cases (4.05 ± 0.32 cm vs. 3.38 ± 0.61 cm). It was observed that, levels of *Maml1* and *Pygo2* mRNA expressions was correlated together significantly ($P = 0.030$). The level of *Maml1* mRNA expression in tumors with only *Maml1* overexpression was lower than that in the *Pygo2*/*Maml1* overexpressed cases (mean ± SD: 3.94 ± 0.80 vs. 4.41 ± 1.06 fold changes). The levels of *Pygo2* mRNA expression in the *Pygo2*/*Maml1* over expressed cases were also interestingly higher than that in the only *Pygo2* overexpressed tumors (mean ± SD: 4.24 ± 1.5 vs. 2.95 ± 0.31 fold changes). Levels of *Maml1* mRNA expression in males were higher than females (mean ± SD: 1.83 ± 0.67 vs. 1.37 ± 0.62 fold changes). In contrast, levels of *Pygo2* mRNA expression in females were higher than males (mean ± SD: 1.34 ± 0.48 vs. 1.22 ± 0.63). Interestingly, we observed that *Pygo2* expression is higher in tumor cells without any lymph node metastasis in comparison with the lymph node metastatic tumors (mean ± SD: 1.70 ± 0.68 vs. 0.76 ± 0.41 fold changes). In contrast, the levels of *Maml1* mRNA expression in lymph nodes metastatic tumors were higher than the cases without lymph nodes metastasis (mean ± SD: 2.01 ± 0.71 vs. 1.37 ± 0.63 fold changes). In the case of tumor location, levels of *Pygo2* and *Maml1* mRNA expression in tumors at middle part of esophagus were higher than the lower part (mean ± SD: 1.69 ± 0.63 vs. 0.73 ± 0.48) and (mean ± SD: 1.95 ± 0.70 vs. 1.29 ± 0.59), respectively.

Discussion

Transcriptional co activators are one of the most important components of transcriptional machineries which mediate gene transcription process (Spiegelman and Heinrich 2004). In the present study we assessed the probable correlation between MAML1 and PYGO2 as the major co activators of NOTCH and Wnt cell signaling pathways, respectively, through expressional analysis in tumors and correlated margin normal esophageal tissues. We showed significant correlation between these markers. There are also significant correlations between the MAML/PYGO2 over expression and some of the clinicopathological features including depth of tumor invasion and tumor size. Regarding the concomitant *Maml1* and *Pygo2* over expression in ESCC cases, we hypothesized that these factors may regulate a cross-talk between NOTCH/Wnt pathways during the ESCC progression. Crosstalk between cell signaling pathways can be categorized into several types including cooperative, separated, and direct crosstalk (Collu et al. 2014). In cooperative mechanism, all of the pathways are united to regulate a specific target while in separated crosstalk, one pathway has a regulatory role on the other one. Direct crosstalk is referred to mutual interaction of different cell signaling pathway transcriptional machineries. The independent crosstalk between NOTCH and Wnt signaling has been shown in self-renewal process of mammary stem cells. In such cells, Wnt pathway inhibits the notch signaling through chromatin remodeling of *Notch3* promoter region using the β-catenin/PYGO2 complex. In this process, chromatin remodeling of *Notch3* promoter region suppresses its expression (Gu et al. 2013). Dishevelled also inhibits NOTCH

through a direct protein-protein interaction (Hayward et al. 2005; Munoz-Descalzo et al. 2010). In line with presented nuclear correlations of these transcription cofactors, It has been reported that the correlation between NOTCH and Wnt pathways is also observed in the cytoplasmic level where the GSK3b as a kinase is involved in several signaling pathways such as β-catenin and NOTCH (Espinosa et al. 2003; Watcharasit et al. 2003). Having applied GSK-3β, Wnt signaling can induce the notch pathway through phosphorylation of NICD and its stabilization (Foltz et al. 2002). GSK-3β is negative regulator of Wnt pathway through phosphorylation and degradation of β-catenin. MAML1 is also negatively regulated by GSK3b. GSK3b inhibits MAML1 through direct interaction with the MAML1 N terminus which is reported to interact with NOTCH and p53 to plays its role in the transcription activity of these factors (Saint Just Ribeiro et al. 2007). This may introduces the regulatory mechanisms for these pathways through cytoplasmic interaction versus nuclear cooperation of their main coactivators.

In this study, the *Pygo2*/*Maml1* overexpressed cells were mostly invaded to the adventitia showing T3 depth of tumor invasion, interestingly. It may rely to the role of cooperation between the concomitant expression of these genes and tumor invasiveness and metastasis. It was observed that, the levels of either *Pygo2* or *Maml1* mRNA expression in tumors with only one gene overexpression were lower than those with concomitant overexpression of the genes. Therefore, it seems that there is a probable feedback between these proteins in ESCC. There is a binding site for the histone acetyltransferase P300 in promoter sequence of *Pygo2* and it has been shown that MAML1 recruits p300 to NOTCH-targeted genes through a direct interaction with the C/H3 domain of p300. Therefore, p300-MAML1 complex may be able to activate the PYGO2 gene transcription through histone acetylation of related chromatin. This acetylation may result in chromatin remodeling of *Pygo2* promoter region leads to activate its expression. On the other hand, Sox9 which is regulated by Wnt pathway has a binding site in the *Maml1* promoter sequence. It seems that these pathways may have a mutual feedback through MAML1 and PYGO2 using the P300 and Sox9 as mediators during the ESCC progression and development (Fig. 2).

Given appropriate knowledge about the interactions between such pathways paves the way for the targeted therapy regarding the cell context. For example, if tumor initiation is due to Wnt activation and NOTCH suppression, using a Wnt ligand inhibitor may be advantageous as a disruptor for the Wnt–NOTCH crosstalk (Chen et al. 2009; Gonsalves et al. 2011). While, targeted therapy for the NOTCH transcription machinery will not result to the proficient consequences because the crosstalk is remained intact.

In conclusion, evaluating of *Maml1* and *Pygo2* concomitant mRNA expression in ESCC patients clarified that their concomitant expression may have important role in ESCC progression and development. This may show a probable cross talk between Wnt and NOTCH pathways through their mastermind transcription factors which can be introduced for ESCC diagnosis and targeted therapy, especially in advanced

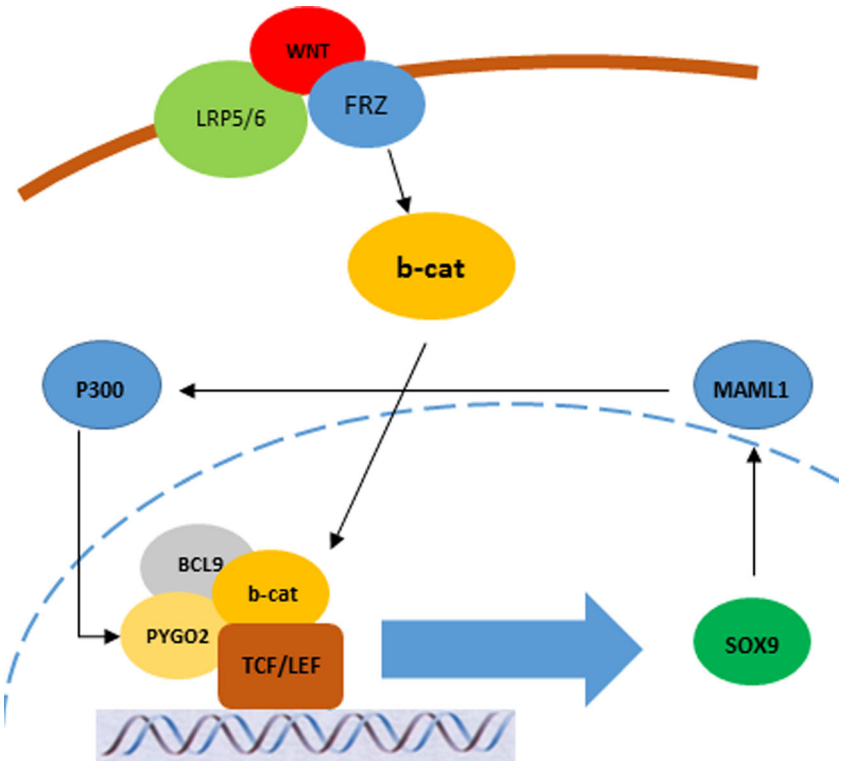

Fig. 2 Probable crosstalk between Wnt and NOTCH pathways through the SOX9 and P300 as the mediators for the PYGO2 and MAML1, respectively

stages of ESCC. To the best of our knowledge, this is the first report describing clinical relevance of concomitant expression of *Maml1* and *Pygo2* in ESCC and evaluating probable crosstalk between related cell signaling pathways. Although, it needs further studies to find more detailed information about this crosstalk in ESCC patients, concomitant evaluation of several pathways shows a better view of molecular mechanisms involved in ESCC system biology.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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