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The homeobox NKX and Neoplasms in Digestive system: a systematic review.

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ABSTRACT: The incidence of digestive system neoplasms is increasing over these years due to the limitation of prompt diagnosis and effective treatment. Homeobox genes are usually associated with development of cancer. Previous studies indicate that homeobox NKX transcription factors are partly involved in the development and progression of tumors in gastrointestinal tract. To summarize the different impact of NKX genes have on the digestive system, the interrelationship between each NKX genes and gastric-derived tumors is analyzed as much detail as possible. The results turn out that some of NKX genes may be used as a target to distinguish the primary site of cancer, or as a biomarker to predict the therapeutic effect and survival which is instructive and meaningful to the future medical managements. This review is a systematic synopsis of research on the homeobox genes NKX and digestive system neoplasms, with the focus on their functions and clinical significance.

KEY WORDS: Homeobox genes; NKX gene family; Gastrointestinal neoplasms; Development; Target.

INTRODUCTION

The homeobox NKX family, which encode several transcription factors, is comprised of the following members: NKX2.1, NKX2.2, NKX2.3, NKX2.4, NKX2.5, NKX2.8, NKX3.1, NKX3.2, NKX6.1, NKX6.2, and NKX6.3. Among these genes, NKX3.1 is an androgen-regulated, prostate-specific homeobox gene whose expression is mainly localized in prostate epithelium. It acts as a transcription factor that has critical function in prostate development and a negative regulator of epithelial cell growth in prostate tissue[1]. NKX3.2 may play a role in skeletal development[2]. Both of them have no connection with gastrointestinal neoplasms, while the rest 9 NKX-related genes are associated with digestive system tumors more or less. The following parts will present the rest 9 NKX-related genes respectively, beginning from NKX2.1.

NKX2.1

NK2 homeobox 1 (NKX2.1), also known as thyroid transcription factor 1 (TTF1), is a 38-kDa homeodomain-containing DNA-binding protein which in humans is encoded by the NKX2.1 gene which location is 14q13[3-4]. This encoded protein was originally identified in follicular cells of the thyroid and was found to bind the thyroglobulin promoter and regulate the expression of thyroid-specific genes[5]. Mutations and deletions in this gene are associated with benign hereditary chorea, congenital hypothyroidism, neonatal respiratory distress, and may be associated with thyroid cancer[6]. Despite the fact that NKX2.1 mostly participates in normal thyroid, brain, and lung development[7], several clinical cases, like ductal carcinoma in situ (DCIS), Merkel cell carcinoma (MCC), benign hereditary chorea (BHC), are recently reported that caused by mutations in the NKX2.1 gene[8-10]. Regarding the gastrointestinal tract, NKX2.1 was found positive in different origin sites with several case reports. As Compérat et al. first reported one case of NKX2.1 positive lung metastasis of colorectal origin[11], Penman et al. claimed NKX2.1 was found positive in less than 10 colorectal adenocarcinomas[12], and some other scientists discovered NKX2.1 positive in 3 gastric adenocarcinomas[13-14]. A previous study directed by Nakamura et al. shows none of NKX2.1 expression come out positive in 10 gallbladder adenocarcinomas[15], while recently Pegolo et al. reported 2 cases of adenocarcinoma of the gastrointestinal tract. One is a gastric adenocarcinoma with liver metastasis, the other is a gallbladder adenocarcinoma discovered during the screening for the colorectal cancer——for the first time that a gallbladder adenocarcinoma documented with diffuse NKX2.1 expression. Then Pegolo et al. conducted an extended study with 10 consecutive primary invasive gallbladder adenocarcinomas, only few NKX2.1 positive cells were detected in 2 out of 10 cases[16]. With plenty research over a long time, the function of NKX2.1 expression in gastrointestinal adenocarcinomas is still uncertain. It may be the result of an interference with the transcriptional control of target genes and other molecular events that leading to the development of the tumors, but further study of NKX2.1 is needed for distinguishing primary tumors from benign and assisting in therapy selection and prognosis prediction.

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NKX2.2

Homeobox protein NKX2.2 is a protein which contains a homeobox domain that in humans is encoded by the NKX2.2 gene. This gene is found on chromosome 20 near NKX2.4, and these two genes appear to be duplicated on chromosome 14 in the form of NKX2.1 and NKX2.8[17]. NKX2.2 is a homeodomain-containing transcription factor that plays an important in neuroendocrine differentiation. It is also considered as a valuable marker for Ewing sarcoma[18], diabetes[19], medulloblastoma and astrocytoma[20]. While in recent years, the major concern of NKX2.2 is its functions in neuroendocrine tumors (NETs). The incidence of NETs has increased over 30 years to 2.5-5 individuals/10000 per year in the US[21]. For most of patients, they can only rely on conservative therapy which is ineffective in shrinking tumors or prolonging life time, while surgery, the only possible curative therapy, is not suitable due to the lymph node involvement or extensive metastasis[22]. The predominant sites of origin for NETs are the gastrointestinal (GI) and bronchopulmonary (BP) systems[23]. The largest subgroups of well-differentiated BP-NETs are typical carcinoids (TCs), while within the GI tract, the small intestine (SI) is the most common site[24]. With the interest of NKX2.2 functions, Ng et al. first published the research that a transgenic zebrafish line with EGFP driven by the NKX2.2a locus identifies endocrine cells in the intestine[25]. Wang et al. carried out a series of studies to ascertain the role of NKX2.2 in normal and neoplastic gut. First, their team demonstrated the distribution of NKX2.2 expression in normal human gut tissue is similar to that in mice, supporting the use of the mouse as a model for studying NE differentiation in the human gut[26]. Then with the use of NKX2.2 mutant mice, the team evaluated NKX2.2 expression in embryonic and adult mouse proximal small intestine by immunofluorescent (IF) analysis, and gave a result that NKX2.2 expression was seen at later embryonic (E) time points and persisted in the adult intestine, which suggested that NKX2.2 is transiently expressed in immature and/or newly differentiated cells. They also detected co-expression of NKX2.2 with 5-HT, GLP-1, gastrin/CCK, somatostatin in adult intestinal crypts, but not along the villi. These results suggest that in the adult intestine, NKX2.2 is transiently expressed in newly differentiated endocrine cells that produce serotonin, GLP-1, gastrin/CCK, and somatostatin. The team analyzed the proximal small intestine in NKX2.2 (-/-) mice, observed that cells producing 5-HT were absent, mRNA levels of tryptophan hydroxylase-1 (Tph1) were almost non-existed, a marked decrease of cells producing gastrin/CCK, GLP-1, substance P and secretin, and a marked increase of cells producing ghrelin. Those indicated that NKX2.2 is critical for the development of several types of gut endocrine cells. Using QRT-PCR, the team analyzed NKX2.2 expression in normal and tumor samples from patients with well-differentiated NETs of ileum and pancreas. Results showed an approximately 20- to 200-fold increase in NKX2.2 expression in ileum, 9- to 900-fold increase in NKX2.2 expression in pancreas when compared with matched normal tissue, proved that NKX2.2 is expressed in NETs derived from the gut. Overall, the researcher detected nuclear expression of NKX2.2 in 83% (24/29) of well-differentiated GI-NETs, including NETs of the stomach, duodenum, ampulla of Vater, pancreas, ileum, and colon. From the readily detection of intense NKX2.2 expression in most human NETs from GI sites, the conclusion were drawn that NKX2.2 is a novel NET-GI marker[26]. For more extended achievement, Wang et al. selected 13 previously undescribed patients with BP-TCs identified at UCSF to ascertain whether the lack of NKX2.2 expression in BP-TCs is useful to distinguish BP- from GI-NETs, while normal human pancreas and a well-differentiated NET of pancreas and one of the ileum were used as controls. It turned out that no evidence of NKX2.2 expression were found in any of the 13 BP-TCs, while it is readily detected in well-differentiated NETs of the pancreas and small intestine on the contrary[27]. In another study about ETS oncogene family transcription factor FEV, Wang et al. found out that NKX2.2, FEV and Asc11 share much in common, for example, their association with tumorigenesis in diverse cancers and promotion of tumor progression in SI-NETs[28]. According to several studies, the homeodomain transcription factor NKX2.2 are thought to regulate development of gut serotonin cells, play important roles in differentiation of normal and neoplastic gut[26], cooperate with FEV and ASC11 in promoting tumor progression in SI-NETs[28], and be a highly sensitive and specific marker of GI-NETs to distinguish BP-NETs [27]. Over last 30 years, the incidence of NETs were still increased and its survival rates were still unsatisfied. Some studies suggested the resection of a GI-NET primary may improve survival[29-30], which means the identification of the primary site is significant for it may effectively help for the selection and evaluation of treatment. According to statistics mentioned before, immunohistochemical staining for NKX2.2 should be applied in clinical management to identify the different form of NETs and guide therapy.

NKX2.3

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NKX2.3 is a member of the NKX family of homeodomain-containing transcription factors, which are implicated in many aspects of cell type specification and maintenance of differentiated tissue functions[31]. Price et al. first realized the existence of NKX2.3 upon isolation of a mouse genomic DNA fragment[32]. By using RT-PCR, Pabst et al. isolates the mouse gene and the corresponding cDNA from embryonic gut tissue, and first reveals the identification and development expression of NKX2.3 gene in the mouse[33]. Over recent years, there are several studies concerning the association between inflammatory bowel diseases (IBD) and NKX2.3 expression. Yu et al. started a series of experiments about NKX2.3, first assumed NKX2.3 may contribute to the pathogenesis of IBD-associated colorectal cancer and sporadic colorectal cancer by regulating the Wnt signaling pathway in 2010[34], then investigated EGR1 gene regulated by NKX2.3 in β cells from both ulcerative colitis (UC) and Crohn's disease (CD) and revealed that NKX2.3 may play different roles in UC and CD pathogenesis in 2012[35]. Meanwhile, the team found out PTPN2 gene regulated by NKX2.3 in CD and suggested it may be a potential target for diagnosis and therapy of IBD[36]. While Arai et al. proposed the idea that the risk haplotype of NKX2.3 confers susceptibility to ulcerative colitis through increasing expression of NKX2.3 mRNA in the colonic mucosa in 2011[37]. Last year, Lu et al. conducted a meta-analysis of 17 studies involving 35358 subjects to demonstrate the contribution of NKX2.3 polymorphisms to IBD[38]. Apart from the concentration on NKX2.3 and IBD that mentioned before, some researchers also notice the impact of NKX2.3 expression on tumors. Colorectal cancer (CRC) is a common gastrointestinal neoplasms which symptoms may include blood in the stool, a change in bowel movements, weight loss, and feeling tired. A clinical study designed by Li et al. was aimed to detect global gene expression of primary advanced colorectal cancer (ACC) patients who have undergone FOLFOX4 chemotherapy. The result shows NKX2.3 highly expressed in chemotherapy sensitive group (experimental group), but its expressions in non-sensitive group (control group) are very low, which means NKX2.3 may be a valuable biomarker that can predict the effects of primary ACC patients who will undergo FOLFOX4[39]. Similarly, Lu et al. examined the genome expression profile in ACC patients who had received FOLFOX4 chemotherapy and get the predictive model involving 7 genes (NKX2.3, FXYD6, TGFB1I1, ACTG2, ANPEP, HOXB8, and KLK11) that had highly accuracy in evaluating chemotherapy sensitivity to the FOLFOX4 regimen[40]. From the research held by Leja et al, NKX2.3 genes are expressed to a lower level in liver metastases than in primary tumors and novel enterochromaffin cells, which implies a essential role in NETs differentiation[41]. In another research, Fotouhi et al. quantified promoter methylation of candidate genes and global methylation in 44 SI-NETs, and observed the reduction of NKX2.3 expression in metastases vs. primary tumors ($P<0.02$), it illustrated that promoter methylation of tumor suppressor genes is associated with suppressed gene expression in SI-NETs[42]. As the association between NKX2.3 expression and IBD is getting clear, more intentions on NKX2.3 and gastric tumors are warranted. The selection of treatment and prediction of prognosis should be guided by more specific evidences and internal mechanisms of NKX2.3.

NKX2.4

NKX2.4 is a member of NKX homeobox family located on chromosome 20p11.22. Its function is not completely thorough with only a few literature were mentioned about NKX2.4 in decades. Wang et al. discussed the conserved linkage of NKX2.2/2.4 in mammals in 2000[43]. Later, Bell et al. analyzed 16 matched normal and tumor tissues for aberrant DNA methylation in 2001. Microarray analysis showed hypomethylation is identified near 14 genes including NKX2.4 and NKX2.5 that encoding predominantly transcription factors[44]. In another study, NKX2.4 was detected as one of previously unreported TCR-translocated oncogene partners in 280 T-cell acute lymphoblastic leukemia (T-ALL) when Le Noir et al. screening for TCRβ and TCRα/δ translocations[45]. Up till the present moment, there is still no evidence to show the interrelation between NKX2.4 and gastrointestinal neoplasms. It is unclear whether NKX2.4 could be a marker for tumors that origin from gastrointestinal? Or would it be an impact factor that affecting the long-time survival and prognosis? Much more investigation is required to "clearly see" whether there is a cause and effect relationship.

NKX2.5

Homeobox protein NKX2.5, also named cardiac-specific homeobox (CSX), is encode by the NKX2.5 gene which plays critical roles in regulating tissue-specific gene expression essential for tissue differentiation and determining the temporal and spatial patterns of development[46]. NKX2.5 is a transcription factor interacted with GATA4 and TBX5 that regulated heart development in humans[47-50], and several latest research show that NKX2.5 mutation may associated with congenital bicuspid aortic valve[51], heterotaxy[52], familial dilated cardiomyopathy and arrhythmias[53]. While more evidence indicate that NKX2.5

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is not only detected in any other organ than heart, Shibata et al. first reported a cell line (NOY1 and NOY2) from human ovarian yolk sac tumor (YST). After analyzing the new cell line, the team suggested NKX2.5 may be a new target in the treatment of ovarian YST[54].It is also assumed that have an effect on T-ALL[55-56]. Regarding the digestive system, Chung et al. declared that NKX2.5 is one of the candidate biomarkers that could be useful in screening for colon cancer[57]. It is also one of the CAMK transport subnetwork upstream genes that may influence the progression and activity of human hepatocellular carcinoma (HCC)[58]. As detection of NKX2.5 in colon cancer and HCC, we should go a step further to figure out whether it exists in other gastric neoplasms and the specific mechanisms of how it worked.

NKX2.8

Homeobox protein 2.8 is a developmental regulator encoded by NKX 2.8 gene which located 14q13.3. In the homeobox family, it is most closely related to NKX2.1 and NKX2.2. According to a plenty of research, NKX2.8 is characterized in bladder cancer[59] and lung cancer[60-61], and may be associated with its poor survival and resistance to chemotherapy. Its downregulation may promotes the progression and poor prognosis in esophageal squamous cell carcinoma (ESCC)[62]. Moreover, Apergis et al. did an extensive research in the relationship between NKX2.8 and HCC. The study established that NKX2.8 is the AFP gene regulator PCF, the HepG2 transcription factor that binds promoter-linked coupling element (PCE), and first demonstrated the association between NKX2.8 and liver development. The result showed that NKX2.8 expression is associated with AFP expression in fetal but not adult liver and in HCC[63]. The discoveries remind us the future areas of investigation should contained the impacts of NKX2.8 overexpression in gastrointestinal neoplasms and the role of NKX2.8 identification in diagnosis and prognosis.

NKX6.1

NKX6.1 (gene symbol NKX6A), a distantly related member of the NKX homeobox gene family, was first identified in islet and insulinoma cell lines[64] by Rudnick et al. in 1994. Then Inoue et al. characterized its genomic structure and chromosomal localization in 1997[65]. Most researchers are focus on the pancreatic with NKX6.1 and believe that NKX6.1 is a homeobox transcription factor participating in the development and regulation of endocrine function of pancreatic islets. In an experiment conducted by Tseng et al, 178 primary and 25 metastatic well-differentiated neuroendocrine tumors (WDNETs) of different origins were analyzed through immunohistochemistry. The statistics show that in the non-tumorous tissues, NKX6.1 expressed in approximately all cells of the pancreatic islets, but non NKX6.1 positive cells were found in lung, stomach, ileum, colon or rectum. While in primary WDNETs, the intensity of NKX6.1 stain was stronger in the pancreatic and duodenal than other organs, which reveals NKX6.1 may can be used as a marker for pancreatic and duodenal WDNETs[66]. While the function of NKX6.1 in gastrointestinal tumors, like HCC, have increasingly become the focus of attention in recent years. HCC is the fifth most common cancer and the third leading cause of cancer-related death worldwide[67-69]. The incidence of HCC has increased over serval years, but unfortunately, the long-term survival of HCC patients are still not ideal. This poor prognosis is mainly attributed to it often being diagnosed at the late clinical stage when surgical treatment and other medical therapies are limited[70]. Serval previous studies show that NKX6.1, a member of the NKX homeodomain-containing family, plays an important roles not only in neuronal tumors[71], cervical tumors[72-74], lymphoma[75] or islet tumor[76-77], but also in HCC and may be a predictive risk factor for unfavorable prognosis in patients with HCC[78-79]. Recently, Huang et al. determined NKX6.1 expression in 12 paired fresh HCC tissues and the adjacent noncancerous liver tissues. The result turns out that NKX6.1 mRNA levels and protein expression were dramatically increased in 8 HCC tissues, and NKX6.1 knockdown inhibited cell invasion and overexpression of NKX6.1 promotes cell invasion. Compared NKX6.1 expression with the clinical pathologic parameters of the HCC patients, the team also discovered that NKX6.1 expression is significantly correlated with tumor size ($p=0.019$), differentiation ($p<0.001$), clinical stage ($p=0.002$), metastasis ($p<0.001$), and relapse ($p=0.007$). Besides, Huang et al. investigated the correlation between NKX6.1 expression and survival in 231 HCC patients, and showed NKX6.1 expression levels can be independent markers for overall survival and contributes to the EMT process in HCC progression[78-79]. NKX6.1 is not only altered in *Helicobacter pylori* infection, incisural antralisation, and intestinal metaplasia[80], we can also see its relationship with gastric cancer from a multicenter prospective cohort study directed by Asada et al. Patients with early gastric cancer, aged 40-80 years, who planned to have or had undergone, ER, enrolled at least 6 months after *Helicobacter pylori* infection discontinued were selected as the research objects. Among 826 patients, 66 of them developed authentic metachronous gastric cancers after the enrolment. The high quartile of methylation levels of three

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preselected genes (miR-124a-3, EMX1 and NKX6.1) had a significant univariate HR and a multivariate-adjusted HR of developing authentic metachronous gastric cancers measuring by quantitative methylation-specific PCR[81]. At same period, a cross-sectional study that also conducted by Asada and Shimazu suggested that NKX6.1 methylation level is closely associated with the risk of gastric cancer incidence[82]. At almost same period, Schneider held a cohort for over 16 years in Colombia, claimed that the level of NKX6.1 DNA methylation was associated with gastric cancer progression, further illustrates that epigenetic modification may affect NKX6.1 expression level in tumor[83]. From the studies we mentioned before, NKX6.1 may be involved in the development of WDNETs, HCC, or gastric cancer, whereas more functional experiments in vivo and vitro are required to confirm the theoretical hypothesis. All those studies showed that NKX6.1 plays an important role in cancer, especially in intestinal tumor, we should pay more deeply attention to its mechanism and figure out how it regulates the tumor progression and its signaling pathway.

NKX6.2

NKX6.2 is a murine-homeobox-containing gene localized distally on chromosome 7. It's a transcription factor with known positive and negative regulatory activities in development and differentiation[84] and seemed to be a tumor suppressor[85]. NKX6.2 is respectively detected in a variety of different neoplasms, like brain tumor[86], bladder cancer[87], renal cell carcinoma[88-89], and lung adenocarcinoma[90]. By so far, no NKX6.2 expression is reported in gastrointestinal neoplasms. It is unknown that whether it is actually not expressed in gastrointestinal tract, or is just not detected yet.

NKX6.3

NKX6.3, a member of the NKX6 subfamily, is known to be an essential regulator in epithelial differentiation. Many studies suggest it is mainly involved in development of the central nervous system (CNS), especially in the hindbrain, and gastrointestinal tract. In 2005, Nelson et al. characterized the expression pattern of NKX6.3 in the hindbrain and gut with the comparison of the two other NKX6 family members, NKX6.1 and NKX6.2[91]. Soon later, Alanentalo et al. did a similar research which indicates NKX6.3 is expressed in duodenal and glandular endoderm and at the end of gestation[92]. In the study directed by Choi et al, the localization of NKX6.3 transcripts is the most distal stomach region, the antrum and pylorus; the expression of NKX6.3 is lower in the intestine. These results implicate NKX6.3 as a selective regulator of G- and D-cell lineages[93]. And lately, Yoon et al. conduct a study aimed to demonstrate NKX6.3 role as an essential tumor suppressor in gastric carcinogenesis. The absence of protein expression and the diminution of DNA copy number and mRNA transcript of the NKX6.3 gene were easily observed in gastric cancers. And NKX6.3 transcriptional factor was concerned to bind specifically to GKN1, a gastric-specific tumor suppressor, and largely increase expression of the latter. Armed with these results, the team suggest that NKX6.3 may decide the fate of gastric mucosal cells and function as a gastric tumor suppressor[94]. For now, NKX6.3 may be a specific screening target for the identification of gastric original diseases and a suppressor for the development of gastrointestinal tumors.

SEARCH STRATEGY AND SELECTION CRITERIA

We systematically searched the literature up to Nov 2015 using PubMed, Web of Science and regional databases. References were identified using the following terms: "NKX", "NKX2", "NKX3", "NKX6", "neoplasms", "gastrointestinal neoplasms", "digestive system neoplasms". All citations are from English-written references. Data restriction were not applied. The coverage of published work is intended to be illustrative rather than absolutely exhaustive.

CONCLUSIONS

The NKX homeodomain proteins are members of a growing family of known vertebrate transcription factors that are believed to play a role in cell type specification and/or maintenance of the differentiated phenotype. The first four originally different NKX genes were identified in Drosophila[95], and subsequently a growing number of NKX homologues in vertebrates. The NKX genes seem to be involved in the specification of positional information[96], as well as in cell type specification and morphogenesis[97-101]. From the articles we mentioned before, some NKX genes, like NKX2.2, are highly sensitive and specific biomarkers for gastrointestinal carcinomas, while others, like NKX2.8, functions are still unclear. To discover more targets of diagnosis and prognosis for patients, more clinical evidenced should be reported and further study of NKX expression in gastrointestinal tract is warranted to validate our hypothesis and provide new insights in therapeutic strategy.

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