Original Article

Hyperpolarization-activated cyclic nucleotide-gated gene signatures and poor clinical outcome of cancer patient

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Background: We investigated the mRNA expression of hyperpolarization-activated cyclic nucleotide-gated genes (HCN1-4) in multiple types and subtypes of cancers.

Methods: We performed a meta-analysis of public microarray data from Oncomine and NextBio Research databases to discover the mRNA expression level of HCN1-4 in cancers. Survival analysis was also used to investigate the correlation between overexpression of HCN gene family with overall survival rate of cancer patients using Kaplan-Meier Plotter database and PROGgene V2 database.

Results: HCN genes (HCN1-4) over-expression and under-expression in multiples types of cancers such as CNS and brain cancer, breast cancer, colorectal cancer, melanoma, and lymphoma were found. HCN1 was significantly correlated with low overall survival of breast cancer [hazard ratio (HR) =7.42, P=0.0019] and colorectal cancer (HR =1.66, P=0.0071) patients. The lower survival rates of lung cancer (HR =2.5, P=0.0107), kidney cancer (HR =1.1, P=0.004) and gastric cancer (HR =1.33, P=0.0037) patients were significantly correlated with the expression of HCN2. HCN3 was significantly correlated to lower survival rates of breast cancer (HR =1.65, P=0.0016), and kidney cancer (HR =1.17, P=0.0049). HCN4 was highly correlated with the lower survival rates of breast cancer of gastric cancer (HR =1.25, P=0.022), lung cancer (HR =5.37, P=0.0433) and ovarian cancer (HR =13.58, P=0.0426).

Conclusions: These data suggested that HCN genes (HCN1-4) are likely to be potential candidates for cancer diagnosis and prognosis.

Keywords: Hyperpolarization-activated cyclic nucleotide-gated channel (HCN channel); cancers; Oncomine; overall survival; diagnosis; prognosis

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Introduction

Cancer is the leading cause of death all over the globe in recent decades. According to WHO, there were 8.2 million people who died from cancer in the year 2012, and in the next two decades this figure will grow to around 22 million (1). Until now, only 30% of cancers could be prevented (1). Commonly, cancer patients would undergo surgery synergistically with chemotherapy and/or radiotherapy, which is painful, and has high mortality rate. According to the U.S. National Cancer Institute, around 200 cancer drugs are commercialized in the market. Nevertheless, researchers in cancer field are still looking to develop new anti-cancer drugs with more specificity and high efficiency (2).

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channel is one of hundreds of intra-membrane ion channels involved in ion transport. HCN channels are

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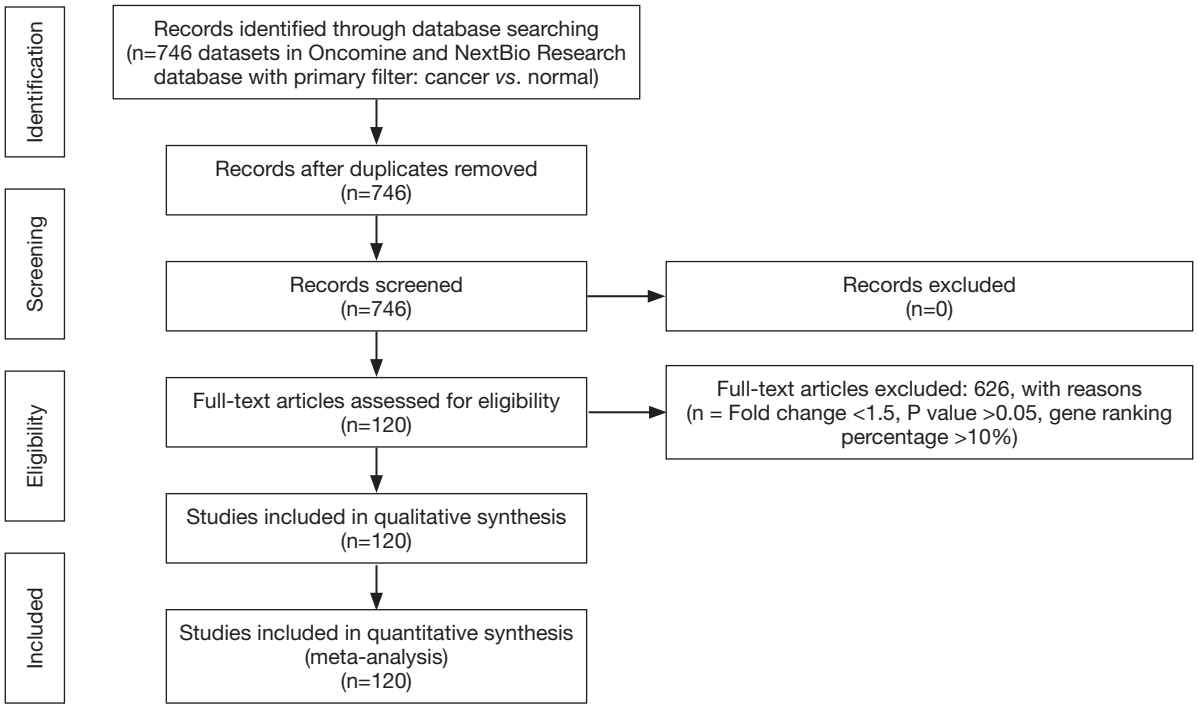

Figure 1 PRISMA 2009 flow diagram. The flow diagram shows screening process and selection criteria for suitable studies of the meta-analysis (21).

encoded by four genes, namely HCN1, HCN2, HCN3 and HCN4 (3). These four genes are predominantly localized and expressed in the heart and the central nervous system (3,4). HCN channels are activated by hyperpolarization, and which permit Na^+ and K^+ to flow inward to the cell (5). HCN channels' main physiological functions are in the heart (6) and the nervous system (4). HCN genes were found to play a role in arrhythmogenic disease and neurological disease (7). The pharmacological properties of these ion channels in cancer are relatively unknown.

Oncomine is a web-based database, which contains more than 700 independent datasets with an estimated 90,000 microarray trials (8,9). The use of Oncomine in several publications confirmed it is a reliable source of clinical datasets (10-16). Oncomine standardizes and organizes the datasets of public cancer microarray data into different cancer types and subtypes (8,9).

NextBio Research database (Illumina INC.) is a web-based platform containing microarray data of more than 20,000 published studies. This online database was introduced by Giovanni Coppola in his book in 2013 (17) and has been used in previous studies (18,19).

In this study, data mining of Oncomine and NextBio Research database was performed to conduct a meta-analysis of HCN gene expression across multiple types and subtypes of cancer. In addition, analysis of survival rate of cancer patients and HCN gene expression was conducted to investigate how these expressions affect the overall survival of cancer patients in the 3 and 5 years' period.

Methods

Data mining

A meta-analysis was performed to analyze the mRNA expression level of HCN gene family in clinical cancer specimens following PRISMA guidelines (20,21) (*Figure 1*, *Tables S1-S9*).

HCN gene (HCN1-4) expression within 17 cancer types was investigated. The mRNA expression of HCN genes in cancerous tissues was grouped by origin of tissue and then compared to normal tissue. Oncomine (www.oncomine.org) and NextBio Research database (https://www.nextbio.com) were used to analyze the mRNA expression of HCN gene family in clinical cancer tissues (22).

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Database search strategy

In this study, the cancer *vs.* normal filter was chosen, which only displayed datasets examining HCN gene mRNA expression in the same origin of tissue. In order to be included in the study, all the data from Oncomine and NextBio research database must satisfy the following threshold: P<0.05, a fold change >1.5 and a gene rank percentile <10% (only applicable to data from Oncomine) (9) (*Figure 1*). Statistical analyses were conducted with Oncomine and NextBio Research default algorithms such as P values, two-tailed Student's *t*-test, and multiple testing corrections. In total, there were 120 studies with 8,471 samples included in this study. All the searches were performed from December 2015 to December 2016.

Survival analysis

The correlation between HCN gene family and overall survival rate was analyzed using Kaplan-Meier plotter (http://kmplot.com/) (23) and PROGgeneV2 (24). Two groups of patients were used for the comparison on survival rates with high and low expression levels of HCN1, HCN2, HCN3 and HCN4 gene.

All the searches were performed from December 2015 to December 2016.

Results

Expression of HCN1 in multiple types and subtypes of cancer

In general, HCN1 gene over-expressed in diverse types of cancer such as colorectal cancer, leukemia, lung cancer, melanoma, and prostate cancer whereas the mRNA expression of HCN1 was under-expressed in breast cancer and bladder cancer. In addition, HCN1 gene also over and under expressed in both lymphoma and pancreatic cancer (*Figure 2*).

The highest expression fold change of HCN1 in cancer and normal matched type tissue was displayed in *Figure 3*. HCN1 expression in brain cancer particular in glioblastoma, glioma was extremely low with the lowest fold change of –151-fold relative to normal brain tissue. However, HCN1 expression was up-regulated in hepatocellular carcinoma and lung cancer with the fold change of 18.8 and 19.9-fold respectively.

Survival analysis of HCN1 expression using Kaplan-Meier plotter and PROGgeneV2 showed that HCN1 had significant correlation with mortality in breast cancer [hazard ratio (HR) =7.42, P=0.0019] and colorectal cancer (HR =1.66, P=0.0071) (*Figure 4*).

Expression of HCN2 in multiple types and subtypes of cancer

In our study, we found that HCN2 gene overexpressed in colorectal cancer, kidney cancer, lung cancer, lymphoma, melanoma, and prostate cancer whereas HCN2 expression level showed both up and down regulation in breast cancer, leukemia, pancreatic cancer, sarcoma, and kidney cancer (*Figure 2*).

HCN2 fold change was calculated and displayed in *Figure 5*. HCN2 expression was down-regulated in esophageal squamous cell carcinoma with fold change of –37.3 and –21.6-fold relative to normal matched type tissue. In contrast, HCN2 was up-regulated in lung cancer, breast cancer, liver cancer, and thyroid cancer with 38.1, 21.4, 13.3, 10.9-fold respectively higher than normal control tissue.

To further investigate the expression of HCN2 and overall survival rate of cancer patients, we used Kaplan-Meier plotter analysis and PROGgeneV2 and found that the lower survival rates of lung cancer (HR =2.5, P=0.0107), kidney cancer (HR =1.1, P=0.004) and gastric cancer (HR =1.33, P=0.0037) had significant correlation with the expression of HCN2 (*Figure 6*). The current data suggested that overexpression of HCN2 may be involved in the particular process of lung cancer. This observation may make HCN2 a potential biomarker for esophageal squamous cell carcinoma, lung cancer, kidney cancer and gastric cancer, breast cancer, liver cancer, and thyroid cancer diagnosis and prognosis.

Expression of HCN3 in multiple types and subtypes of cancer

Our data showed that HCN3 gene over-expressed in breast cancer, kidney cancer, lung cancer, liver cancer, gastric cancer, ovarian cancer, bladder cancer, kidney cancer whereas HCN3 was under-expressed in prostate cancer (*Figure 2*).

HCN3 was under expressed in many subtypes of brain cancer such as primary tumor dermal neurofibroma, cultured plexiform neurofibroma-derived Schwann cell, pediatic tumor tissue ependymoma, pediatic tumor tissue anaplastic astrocytoma with fold change of –4.5, –3.5,

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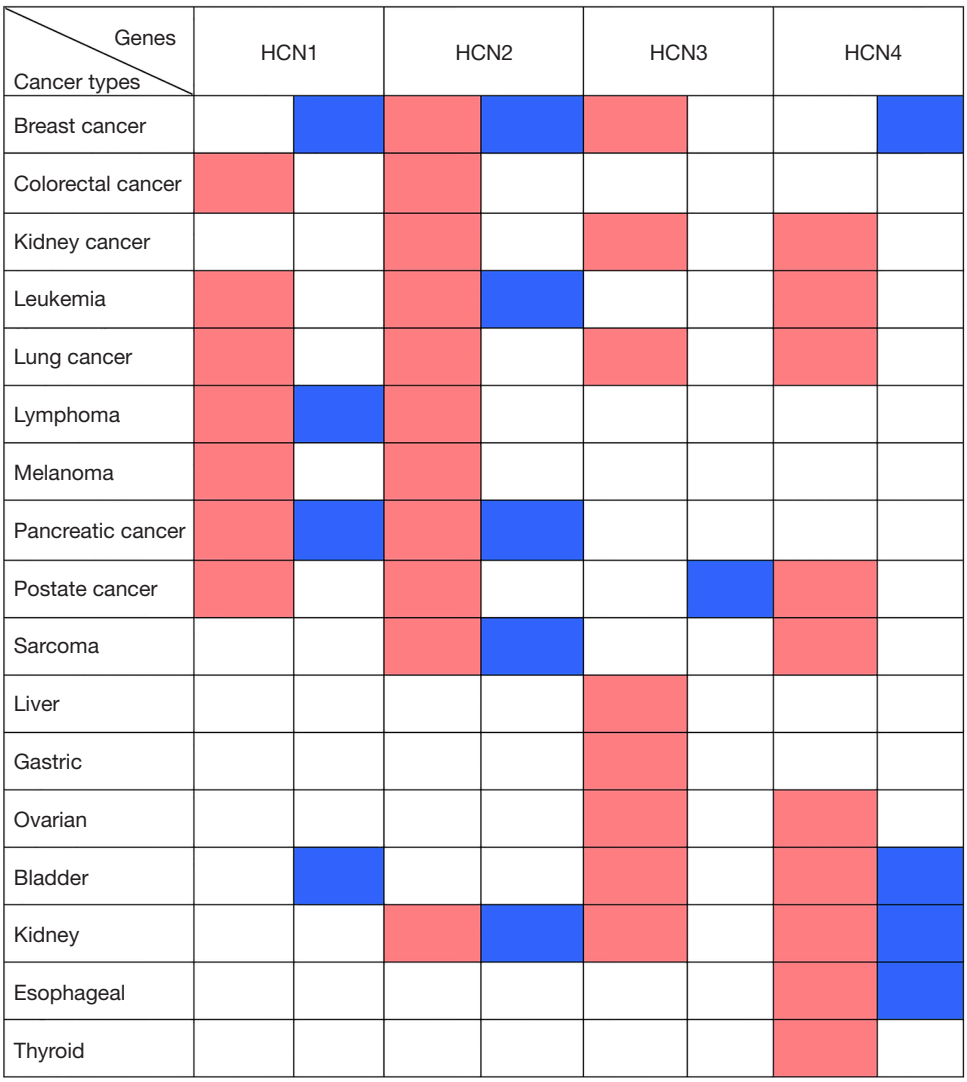

Figure 2 Expression of hyperpolarization-activated cyclic nucleotide-gated genes in multiple types of cancer. Expression of HCN genes in 17 types of cancers compared to normal matched type tissue controls. The color correlates with over and under expression of genes in specific cancer. Red color represents for over expression and blue color represents for under expression. The search criteria threshold was set at P<0.05 with fold change >1.5 and gene rank percentile <10% for screening microarray datasets of cancer versus normal cases. HCN, hyperpolarization-activated cyclic nucleotide-gated.

–3.2, –3-fold respectively compared to normal matched type tissue (*Figure* 7). In contrast, HCN3 over-expressed in liver and lung cancer tissue with fold change of 3.9 and 3.6 respectively, relatively to normal matched type sample (*Figure* 7).

In addition, Kaplan-Meier plotter and PROGgeneV2 analysis showed overexpression of HCN3 in breast cancer was significantly correlated with lower survival rates and poor prognosis value of breast cancer (HR =1.65, P=0.0016), kidney cancer (HR =1.17, P=0.0049) but higher survival rate and good prognosis value in lung cancer (HR =0.33, P=0.0272) and ovarian cancer (HR =0.53, P=0.0386) patients (*Figure 8*). This result may indicate HCN3 as a potential biomarker for diagnosis and prognosis of brain cancer, breast cancer, kidney cancer, lung cancer and ovarian cancer.

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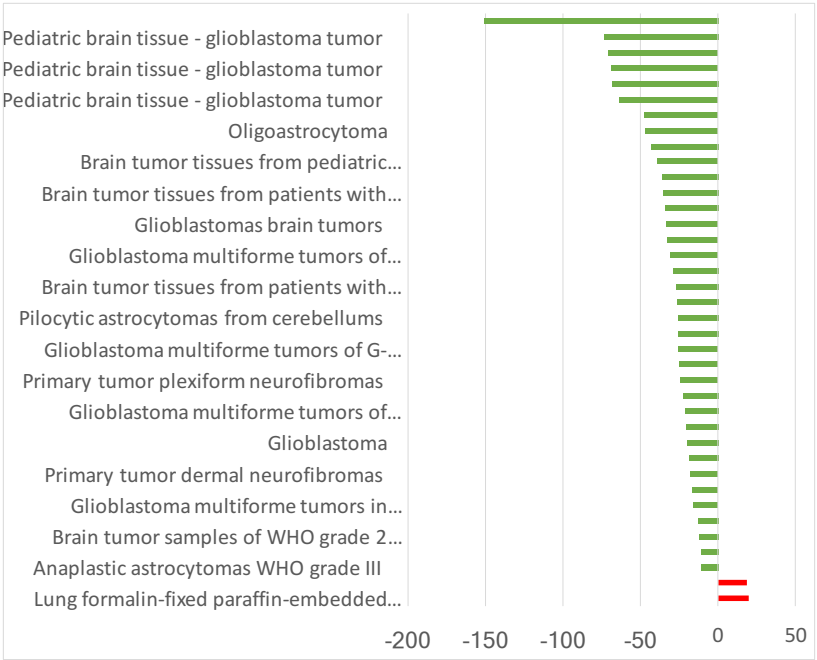

Figure 3 Expression of HCN1 genes in multiple subtypes of 17 cancers. Only two datasets shown over expression of HCN1 in cancer while the rest of cancer subtype had under expression of HCN1.

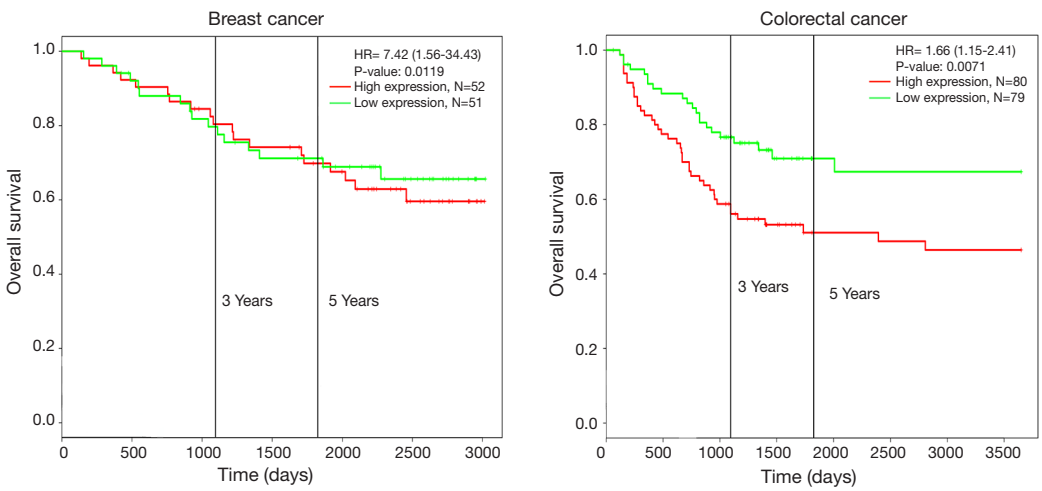

Figure 4 HCN1 mRNA expression and overall survival patient with breast cancer and colorectal cancer. High expression of HCN1 results in poor survival rate of patient in 3 and 5 years' period. P<0.05 means statistically significant difference.

Expression of HCN4 in multiple types and subtypes of cancer

HCN4 gene was found over-expression in kidney cancer, leukemia, lung cancer, sarcoma, ovarian cancer, and thyroid cancer whereas it under expressed in breast cancer. In addition, both over and under expression of HCN4 were found in bladder cancer, kidney cancer, and esophageal cancer (*Figure 2*).

HCN4 was under-expressed in stage I and II endometrial carcinoma with the fold change extremely low (–157 and

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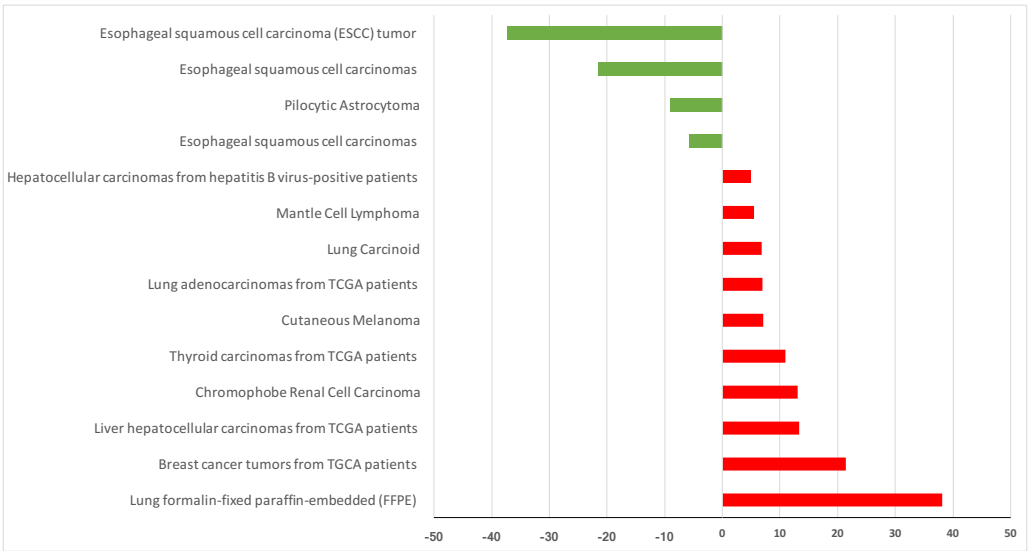

Figure 5 Expression of HCN2 genes in multiple subtypes of 17 cancers. High expression of HCN2 was recorded in multiple subtypes of cancer.

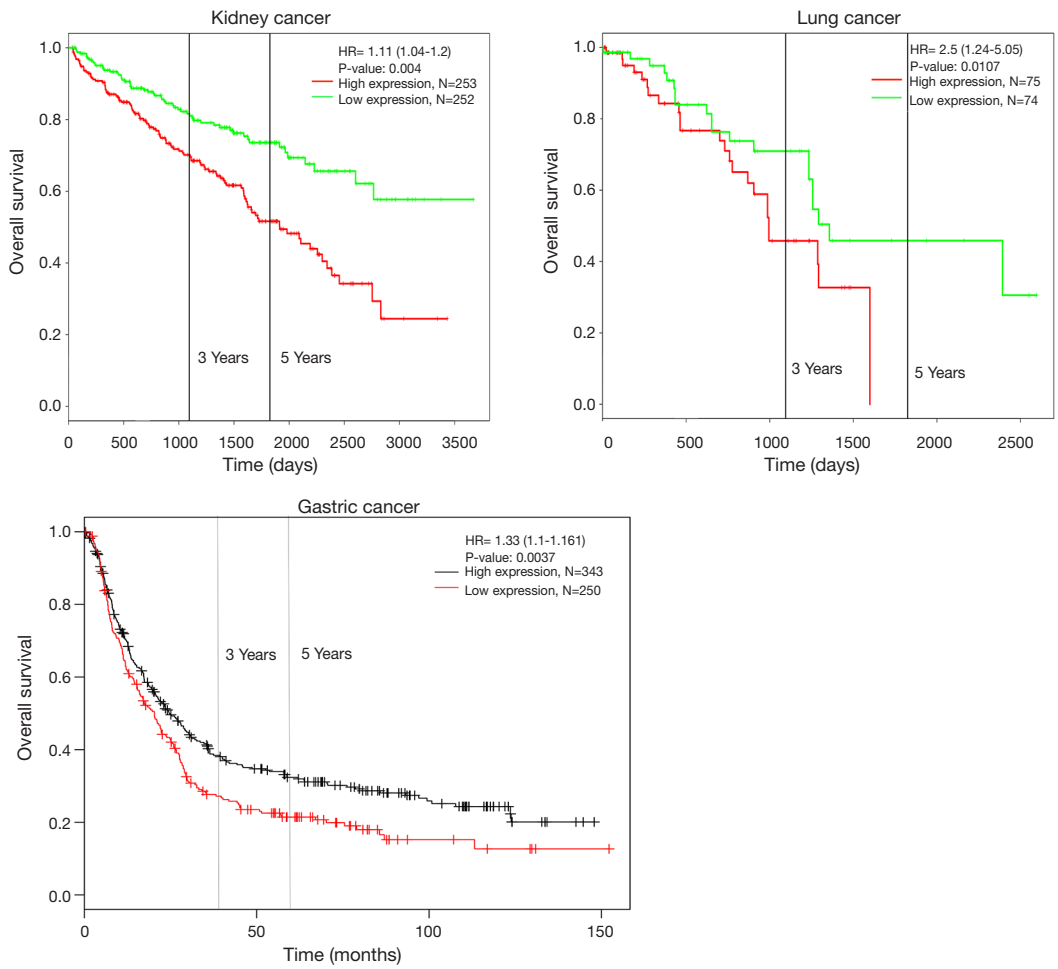

Figure 6 HCN2 mRNA expression and overall survival patient with kidney cancer, lung cancer and gastric cancer. High expression of HCN2 results in poor survival rate of patient in 3 and 5 years' period. P<0.05 means statistically significant difference.

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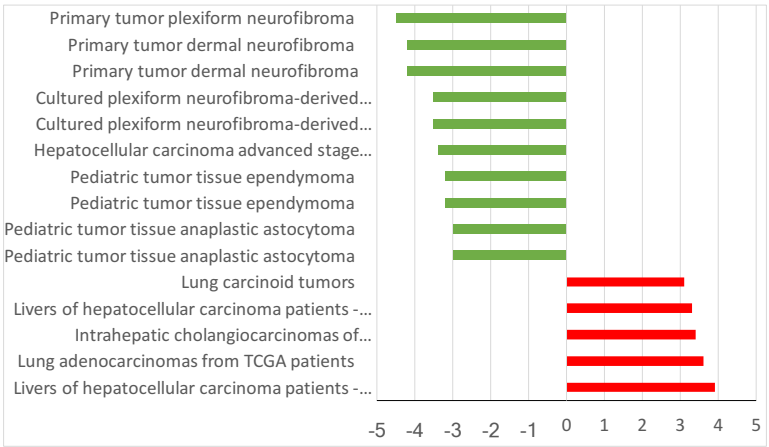

Figure 7 Expression of HCN3 genes in multiple subtypes of 17 cancers. High and low expression of HCN3 was recorded in multiple subtypes of cancer.

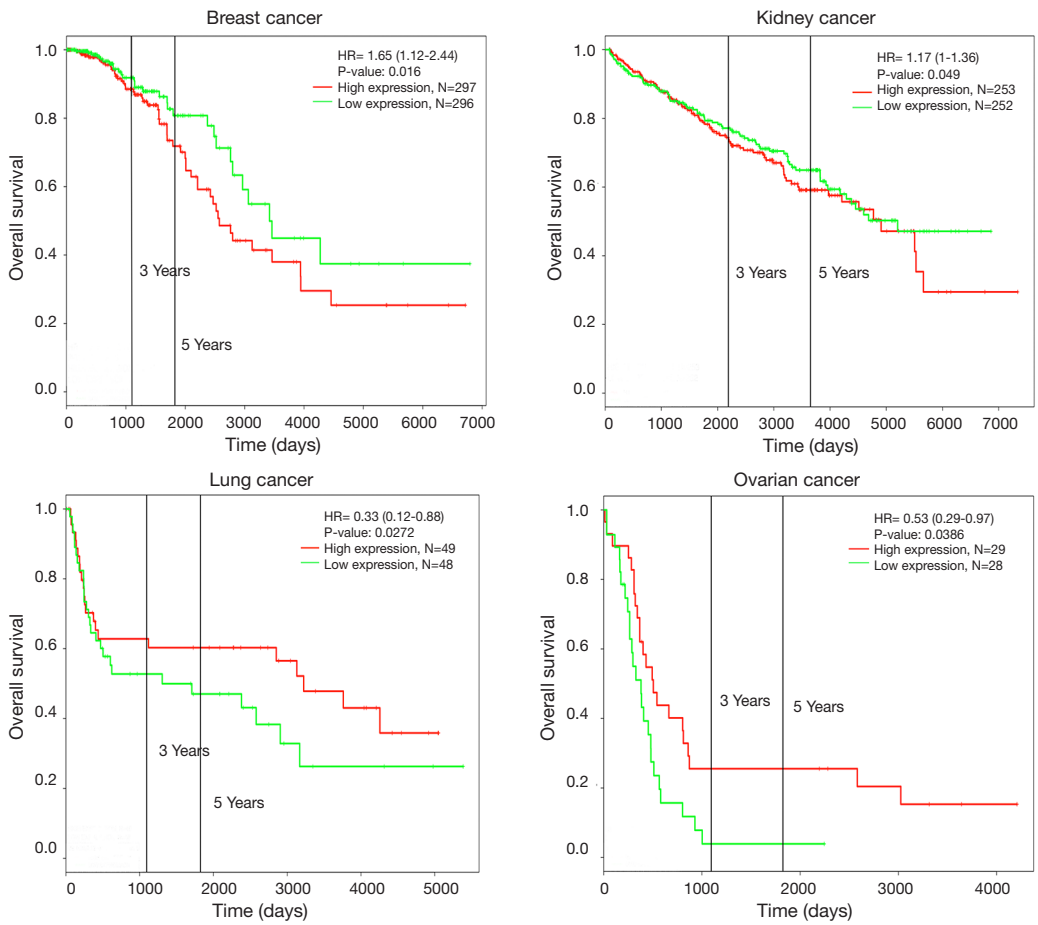

Figure 8 HCN3 mRNA expression and overall survival patient with breast cancer, kidney cancer, lung cancer and ovarian cancer. High expression of HCN3 results in poor survival rate of patient in 3 and 5 years' period. P<0.05 means statistically significant difference.

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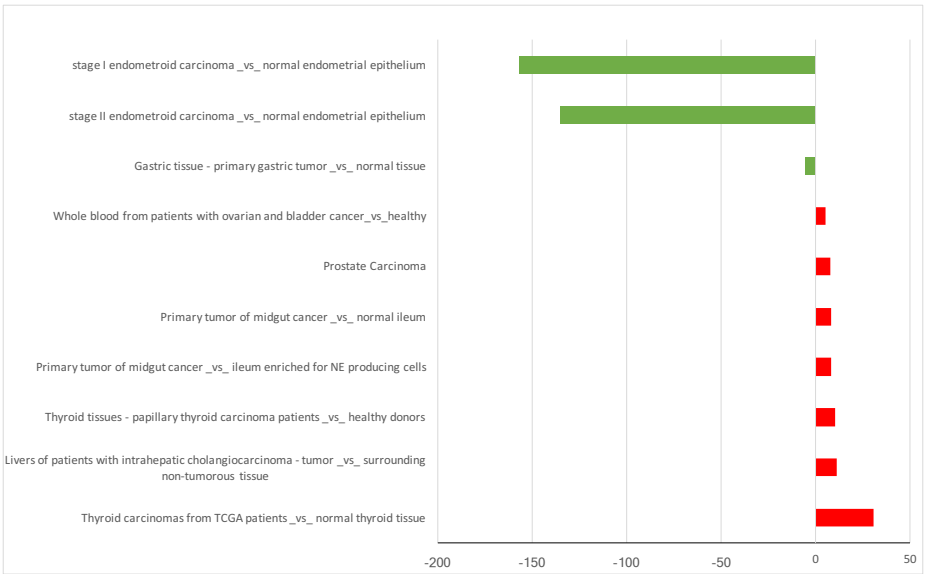

Figure 9 Expression of HCN4 genes in multiple subtypes of 17 cancers. High and low expression of HCN4 was recorded in multiple subtypes of cancer. HCN4 shown extremely low expression level in stage I endometrioid carcinoma and high expression in thyroid carcinoma.

–135-fold). In contrast, HCN4 was over expressed in Thyroid carcinomas, Thyroid tissues-papillary thyroid carcinoma, liver cancer, and prostate cancer with fold change of 30.4, 10, 11.2, and 7.8-fold respectively (*Figure 9*).

Kaplan-Meier plotter analysis showed that upregulation of HCN4 was highly correlated with the lower survival rates of patients with gastric cancer (HR =1.25, P=0.022), lung cancer (HR =5.37, P=0.0433) and ovarian cancer (HR =13.58, P=0.0426) but higher survival rate in patient with breast cancer (HR =0.8, P=0.00016) (*Figure 10*). From these results, HCN4 can be considered as the potential marker in breast cancer, gastric cancer, lung cancer and ovarian cancer diagnosis, thyroid carcinomas.

Discussion

In this study, we showed that HCN family members (HCN1, HCN2, HCN3, HCN4) overexpressed in numerous cancerous tissue relative to normal matched tissue. The increased expression of these four genes in multiple types and subtypes of cancer was also significantly correlated with low and high survival rates of cancer patients. This correlation suggests that HCN genes might play a key role in cancer particularly in brain cancer, lung cancer, liver cancer, esophageal cancer, thyroid cancer, ovarian cancer which had notably high fold change compared to normal matched type tissue. However, further study is required to confirm the mechanism of how HCN genes play a role in cancer.

In neuropathic pain, lacking of HCN1 gene expression by genetic deletion showed mitigation in neuronal damage (25). Another research showed that HCN1 deficiency caused epilepsy, ataxia and learning compromise (7). In a recent study, HCN1 showed under-expression with fold change of 0.65 in breast cancer cells after Maitake D-Fraction treatment (26). Single nucleotide polymorphism of HCN1 was found association with shorter survival of breast cancer patient (27). Moreover, inhibiting of HCN channel functions in embryonic stem cells by ZD7288, a HCN channels blocker, and cesium revealed that cell proliferation was decreased under the effects of these two drugs (28). HCN3 gene was also implied as the potential target for tumor suppression (28). In our findings, HCN3 also showed overexpression in multiple types of cancers such as breast cancer, and liver cancer. Therefore, we speculate that HCN3 is likely to be a candidate to study cancer cell proliferation and cancer cell cycle.

The HCN gene is commonly located in ventricular myocytes and neuron cells. Previous studies on the roles of the HCN channels were primarily focused on neurological diseases such as epilepsies and neuropathic pain disorders and cardiac related diseases (7,29,30). HCN channel functions are largely unknown in cancer. HCN channels (HCN1-4) have been known to allow the flow of Na^+ and K^+ ions (1:4 ration) inward and outward of the cell, which

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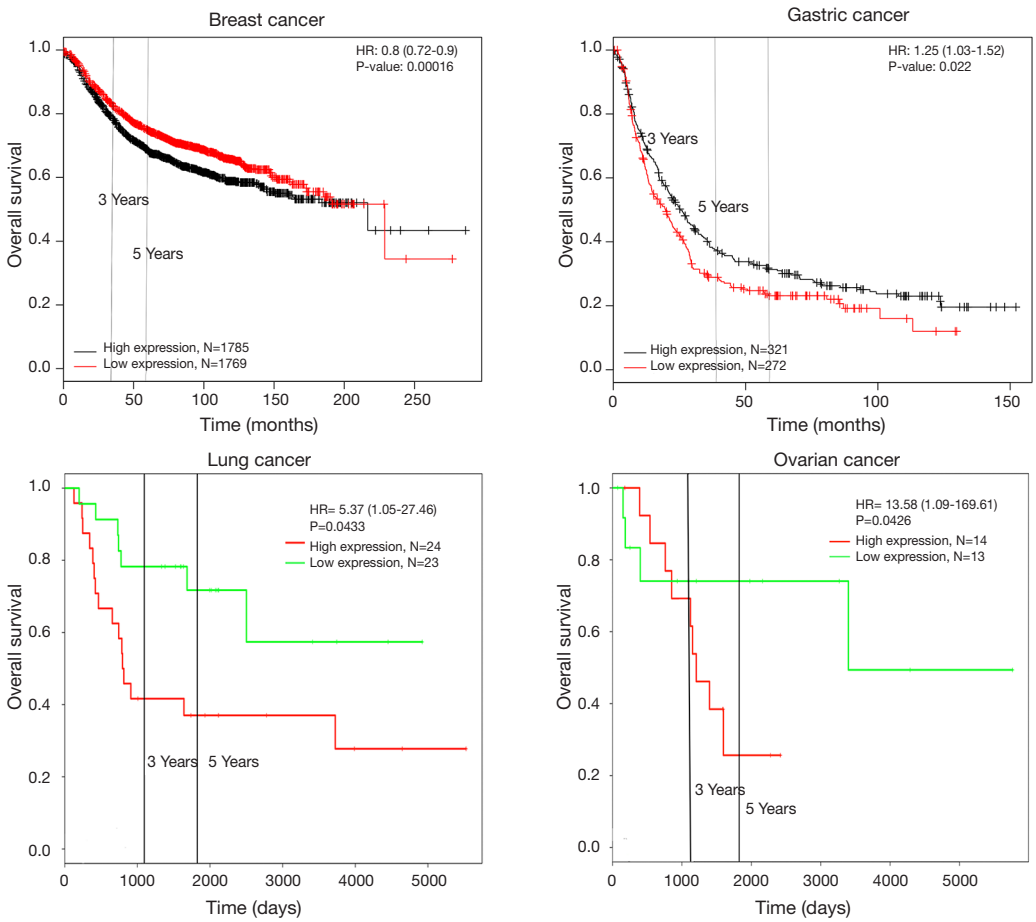

Figure 10 HCN4 mRNA expression and overall survival patient with breast cancer, gastric cancer, lung cancer and ovarian cancer. High expression of HCN4 dramatically declined the survival rate of patient in 3 and 5 years' period. P<0.05 means statistically significant difference.

creates a hyperpolarization activated current named Ih. This current was showed to participate in regulating the heart rate and the firing of neurons. Moreover, HCN channels also play a role in the determination of resting membrane potential, dendritic integration, synaptic transmission and learning (7). HCN2 roles in inflammatory and neuropathic pain have been uncovered recently (31). Intriguingly, apart from the permeability of Na^+ and K^+ inward cell, HCN2 and HCN4 also allow $Ca2^+$ ion into the cell (32). This happened due to the dephosphorylation of Thr549 within the regulatory region of HCN2; and calcium ion influx causes cell apoptosis due to cytotoxicity (33). cAMP was acknowledged to modulate HCN2 in gating activity (34,35). Moreover, in non-small cell lung carcinomas, HCN2 has also been triggered by PKC inhibitors such as staurosporine (STS) or PKC412 and under expression of HCN2 can prevent cell apoptosis (36). As a consequence, if HCN2 is mutated or overexpressed in cancer cells, it can lead to cancer cell not going through apoptosis. Thus, HCN2 expression is crucial and likely to be a potential target for cancer treatment via inhibiting of HCN2 expression.

The current study is the pioneer meta-analysis research about HCN gene expression in multiple types and subtypes of cancer. HCN1-4 could have potency as biomarker for cancer disease diagnosis and prognosis. Further study on HCN genes and specific types of cancer as suggested in the present study may help to reveal the underlying molecular mechanism of these genes in cancer.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2017.07.22). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Supplementary

Table S1 HCN1 expression in multiple types of cancer from NextBio Research database

Cancer type	Subtype	P value	Fold change (cancer/normal)	N	References
Brain cancer	Anaplastic astrocytomas WHO grade III	5.80×10^{-5}	−11.0	5	(37)
	Astrocytoma grade III	0.0162	4.7	3	(38)
	Brain from newly diagnosed WHO grade 4 glioblastoma patients	3.00×10^{-20}	−151.0	40	(39)
	Brain from oligodendroglioma	5.00×10^{-8}	−7.8	50	(40)
	Brain glioma stem cells	0.0171	−20.6	N/A	(41)
	Brain tissue—astrocytomas	2.70×10^{-33}	−9.8	148	(42)
	Brain tissue—tumors with mixed histology	3.10×10^{-5}	−8.8	11	(42)
	Brain tumor samples of WHO grade 2 diffusely infiltrative astrocytoma	9.40×10^{-5}	−12.1	5	(43)
	Brain tumor samples of WHO grade 2 ependymoma	0.0274	−7.7	4	(43)
	Brain tumor samples of WHO grade 2 oligodendroglioma	0.0165	−6.5	5	(43)
	Brain tumor tissues from adult patients with high grade glioma	0.0028	−34.6	7	(43)
	Brain tumor tissues from patients with atypical teratoid-rhabdoid tumor	0.0051	−35.4	17	(43)
	Brain tumor tissues from patients with group 3 medulloblastoma	0.0173	−6.0	4	(43)
	Brain tumor tissues from patients with group 4 medulloblastoma	0.0017	−16.5	7	(43)
	Brain tumor tissues from patients with posterior fossa group B ependymoma	0.0163	−5.1	26	(43)
	Brain tumor tissues from patients with sonic hedgehog medulloblastoma	0.0017	−27.0	8	(43)
	Brain tumor tissues from patients with supratentorial ependymoma	0.018	−47.3	9	(43)
	Brain tumor tissues from pediatric patients with high grade glioma	0.0128	−39.2	14	(43)
	Classic medulloblastomas	0.0114	−1.8	N/A	(44)
	Giant cell glioblastoma	0.0007	−36.4	5	(45)
	Glioblastoma	4.60×10^{-6}	−43.2	1	(45)
	Glioblastoma multiforme grade IV	6.00×10^{-10}	−4.4	6	(46)
	Glioblastoma multiforme solid tumor	0.0016	−1.7	19	(47)
	Glioblastoma multiforme tumors in pediatric cases	0.0041	−16.1	25	(48)
	Glioblastoma multiforme tumors of classical subtype	2.50×10^{-14}	−30.7	N/A	(48)
	Glioblastoma multiforme tumors of G-CIMP (–) subtype	9.80×10^{-12}	−25.1	N/A	(48)
	Glioblastoma multiforme tumors of G-CIMP (+) subtype	3.80×10^{-16}	−25.2	N/A	(48)
	Glioblastoma multiforme tumors of mesenchymal subtype	5.60×10^{-13}	−32.6	N/A	(48)
	Glioblastoma multiforme tumors of neural subtype	4.00×10^{-16}	−12.7	N/A	(48)
	Glioblastoma multiforme tumors of patients surviving less than 3 months	7.00×10^{-18}	−28.9	N/A	(48)
	Glioblastoma multiforme tumors of patients surviving longer than 36 months	7.60×10^{-17}	−25.8	N/A	(48)
	Glioblastoma multiforme tumors of proneural and G-CIMP (–) subtypes	2.60×10^{-15}	−22.3	N/A	(48)
	Glioblastoma multiforme tumors of proneural and G-CIMP (+) subtypes	2.10×10^{-16}	−26.8	N/A	(48)
	Glioblastoma multiforme tumors of proneural subtype	2.90×10^{-15}	−21.0	N/A	(48)
	Glioblastomas brain tumors	0.0115	−33.2	N/A	(49)
	Pediatric brain tissue—glioblastoma tumor	7.40×10^{-14}	−69.0	12	(50)
	Pediatric brain tissue—glioblastoma tumor	9.80×10^{-7}	−68.5	12	(50)
	Pediatric brain tissue—glioblastoma tumor	6.30×10^{-13}	−73.3	12	(50)
	Pediatric brain tissue—glioblastoma tumor	5.10×10^{-6}	−70.5	12	(50)
	Pediatric brain tissue—glioblastoma tumor	4.00×10^{-12}	−63.6	12	(50)
	Pediatric cerebellum from medulloblastoma patients (aged 114–155 months)	0.0437	−4.2	7	(50)
	Pediatric cerebellum from medulloblastoma patients (aged 27–51 months)	0.0028	−6.7	8	(51)
	Pediatric cerebellum from medulloblastoma patients	1.70×10^{-5}	−6.7	19	(51)
	Pilocytic astrocytomas from cerebellums	5.20×10^{-21}	−26.3	35	(52)
	Primary melanoma—superficial spreading melanoma	0.001	2.3	46	(53)
	Primary neuroblastoma with 11q deletion	0.0457	−2.0	8	(54)
	Primary tumor dermal neurofibromas	0.0104	−17.6	13	(55)
	Primary tumor malignant peripheral nerve sheath tumors	0.01	−11.4	13	(55)
	Primary tumor plexiform neurofibroma	0.0033	2.9	13	(56)
	Primary tumor plexiform neurofibromas	0.0113	−24.4	6	(55)
	Diffusely infiltrating astrocytic gliomas WHO grade II	0.0132	−7.6	5	(43)
	Oligoastrocytoma	3.60×10^{-6}	−47.0	2	(45)
Kidney cancer	Adrenal tissue from pheochromocytoma patients with HIF2A mutation	0.0012	2.5	1	GSE50442
	Adrenal tissue from pheochromocytoma patients with PHD2 mutation	8.40×10^{-5}	−4.2	1	GSE50442
Breast cancer	Breast cancer tumors from TGCA patients	1.20×10^{-16}	−3.0	1,018	TCGA
Colorectal cancer	Colon carcinoma LS174T cells (undifferentiated)	3.70×10^{-6}	9.6	3	(45)
Skin cancer	Skin tissue—primary Merkel cell carcinoma non-viral origin	0.0274	3.0	7	(57)
Stomach cancer	Stomach cancer with diffuse morphology stage IIIA	0.0026	1.5	6	(58)
Liver cancer	Hepatocellular carcinoma etiology alpha-1 antitrypsin deficiency	0.0065	18.8	N/A	(59)
	Intrahepatic cholangiocarcinomas of inflammation subtype	0.0174	−1.7	57	(60)
	Intrahepatic cholangiocarcinomas of proliferation subtype	0.0063	−2.0	92	(60)
Lymphoma	Large granular lymphocyte leukemia cells wild type STAT3	0.0315	4.3	1	(61)
Lung cancer	Lung adenocarcinomas from TCGA patients	9.00×10^{-8}	7.3	454	TCGA
	Lung FFPE—tumor	1.10×10^{-5}	19.9	4	(62)
Melanoma	Melanoma skin biopsies—primary melanoma	0.0004	2.0	46	(53)
Pancreatic cancer	Pancreatic ductal adenocarcinoma tissues	2.80×10^{-6}	−2.6	6	(63)
Thyroid cancer	Papillary thyroid carcinomas with BRAF mutation	3.10×10^{-7}	−4.2	14	GSE54958
	Papillary thyroid carcinomas without BRAF mutation	5.20×10^{-7}	−4.0	11	GSE54958

FFPE, formalin-fixed paraffin-embedded.

Table S2 HCN1 expression in multiple types of cancer from Oncomine database

Cancer	Subtype	N (case)	Expression	P value (cancer/normal)	*t*-test (cancer/normal)	Fold (cancer/normal)	Gene ranking (%)	Database
Brain and CNS cancer	Anaplastic oligoastrocytoma	54	↓	2.25×10^{-4}	–5.732	–4.034	417 (in top 3%)	Cancer Res 2005/10/01 (64)
	Oligodendroglioma	54	↓	0.001	–4.643	–3.418	746 (in top 6%)	Cancer Res 2005/10/01 (64)
	Glioblastoma	54	↓	6.71×10^{-6}	–6.333	–4.106	1,116 (in top 8%)	Cancer Res 2005/10/01 (64)
	Anaplastic oligodendroglioma	54	↓	0.005	–5.540	–5.176	1,437 (in top 10%)	Cancer Res 2005/10/01 (64)
	Glioblastoma	NA	↓	9.08×10^{-6}	–20.032	–18.892	675 (in top 4%)	J Clin Oncol 2008/06/20 (65)
	Anaplastic oligoastrocytoma	180	↓	2.12×10^{-6}	–5.321	–5.462	1,592 (in top 9%)	Cancer Cell 2006/04/01 (40)
	Glioblastoma	180	↓	8.01×10^{-14}	–9.613	–20.247	1,615 (in top 9%)	Cancer Cell 2006/04/01 (40)
Breast cancer	Invasive ductal and lobular carcinoma	593	↓	2.65×10^{-4}	–8.273	–2.809	957 (in top 5%)	TCGA 2011/09/02
	Male breast carcinoma	593	↓	0.003	–5.409	–2.113	2,024 (in top 10%)	TCGA 2011/09/02
	Invasive breast carcinoma	158	↓	0.002	–7.245	–2.780	1,530 (in top 9%)	Breast Cancer Res Treat 2011/03/04 (66)
Colorectal cancer	Rectal mucinous adenocarcinoma	237	↑	4.13×10^{-7}	8.326	3.800	225 (in top 2%)	TCGA Colorectal 2011/09/08
Lymphoma	Marginal zone B-cell lymphoma	27	↑	0.007	3.171	1.576	388 (in top 3%)	J Invest Dermatol 2003/05/01 (67)
	Unspecified peripheral T-cell lymphoma	60	↓	5.58×10^{-13}	–9.897	–1.584	249 (in top 2%)	J Clin Invest 2007/03/01 (68)
	Angioimmunoblastic T-cell lymphoma	60	↓	1.39×10^{-4}	–7.676	–1.967	1,817 (in top 10%)	J Clin Invest 2007/03/01 (68)
Melanoma	Cutaneous melanoma	87	↑	0.007	2.763	2.479	1,548 (in top 8%)	BMC Med Genomics 2008/04/28 (69)

Table S3 HCN2 expression in multiple types of cancer from NextBio Research database

Cancer subtype	P value	Fold change	N	References
Lung FFPE	0.0012	38.1	4	(62)
Breast cancer tumors from TGCA patients	4.00×10^{-58}	21.4	1,018	TCGA
Liver hepatocellular carcinomas from TCGA patients	2.00×10^{-17}	13.3	165	TCGA
Thyroid carcinomas from TCGA patients	1.20×10^{-13}	10.9	500	TCGA
Lung adenocarcinomas from TCGA patients	8.20×10^{-19}	6.9	454	TCGA
Hepatocellular carcinomas from hepatitis B virus-positive patients	3.40×10^{-6}	5	10	(70)
B cells CD19+ from peripheral blood—chronic lymphocytic leukemia with mutated IGVH	0.0002	4.6	4	GSE70830
Actinic keratosis cells	7.80×10^{-7}	3.3	5	GSE42677
Prostate adenocarcinomas from TCGA patients	6.70×10^{-12}	3.3	259	TCGA
Seminoma cells	0.0006	3.1	23	(71)
Lung squamous cell carcinomas from TGCA patients	2.80×10^{-9}	2.8	482	TCGA
Thyroid tissues—papillary thyroid carcinoma patients	0.006	2.8	18	(72)
Lung adenocarcinoma primary tumors	0.0044	2.8	6	(73)
Bladder cancer tumors from TCGA patients	0.0025	2.6	223	TCGA
Livers of hepatocellular carcinoma patients	0.0004	2.5	12	GSE56545
Oncocytoma	1.40×10^{-9}	2.2	7	(74)
iPS cells derived from bone marrow mononuclear cells of CML patient	0.0438	2.1	1	(75)
Pancreatic neuroendocrine tumors	0.0024	2	6	(76)
Chromophobe renal tumor	0.005	2	6	(74)
Renal tissue—ChRCC	9.70×10^{-5}	2	10	(77)
Primary melanoma (before treatment)	0.0253	2	31	(77)
Primary melanoma culture	0.01	1.9	2	(78)
Lymphoma cells of lymph nodes of tcr-cHL	0.0011	1.7	4	(79)
Adrenal tissue from pheochromocytoma patients with HIF2A mutation	0.0054	1.6	1	GSE50442
Nodular lymphocyte predominant Hodgkin lymphoma	0.0422	1.6	5	(80)
Mesenchymal stem cells 7–9 passages—large granular lymphocyte leukemia	0.0332	1.6	2	(81)
Prostate cancer stage T2	0.0051	1.5	3	(82)
Breast cancer Luminal A subtype	0.0005	1.5	39	(83)
Familial pancreatic intraepithelial neoplasias	0.0439	–1.6	13	(84)
Metastatic breast tumor	0.0298	–1.6	5	(85)
Pancreatic intraepithelial neoplasias of family C	0.0363	–1.6	2	(84)
Intestinal cancer stage IB	0.0055	–1.7	10	(58)
High-stage neuroblastoma	0.0076	–1.7	12	(86)
Infiltrating ductal mammary carcinoma	1.10×10^{-27}	–1.7	68	(87)
Pancreatic intraepithelial neoplasias of family X	0.0349	–1.7	7	(84)
Esophageal squamous cancer cell line	0.0021	–1.7	20	(88)
Brain tissue—glioblastoma tumors	1.40×10^{-5}	–1.8	48	(89)
Glioblastomas brain tumors	1.50×10^{-7}	–1.9	34	(49)
Low-stage neuroblastoma	0.0046	–1.9	6	(86)
Brain tumor tissues from patients with sonic hedgehog medulloblastoma	0.0034	–1.9	8	(90)
Brain tumor tissues from patients with posterior fossa group B ependymoma	1.20×10^{-9}	–2.1	26	(90)
Brain tumor tissues from patients with posterior fossa group A ependymoma	7.00×10^{-10}	–2.2	29	(90)
Molecular profiling of PBMC from T-cell LGL	0.001	–2.2	30	(91)
Pilocytic astrocytoma tumor	0.0319	–2.5	6	GSE12657
Brain tumor tissues from patients with atypical teratoid-rhabdoid tumor	1.20×10^{-9}	–2.5	17	(90)
Brain tumor tissues from adult patients with high grade glioma	7.80×10^{-6}	–2.6	7	(90)
Glioblastoma tumor	0.0138	–2.9	7	GSE12657
Hepatocellular carcinoma early stage tissue	0.0011	–3	13	(92)
Intrahepatic cholangiocarcinomas of proliferation subtype	0.0441	–3.4	92	(60)
Plasma cells from multiple myeloma patients	0.0009	–4.2	12	GSE9656
ESCC	0.001	–5.8	4	(93)
ESCC	5.90×10^{-12}	–21.6	3	(93)
ESCC tumor	5.70×10^{-23}	–37.3	3	(94)

FFPE, formalin-fixed paraffin-embedded; ChRCC, chromophobe renal cell carcinoma; ESCC, esophageal squamous cell carcinoma.

Table S4 HCN2 expression in multiple types of cancer from Oncomine database

Cancer	Subtype	N (case)	Expression	P value (cancer/normal)	*t*-test (cancer/normal)	Fold (cancer/normal)	Gene ranking (%)	Database
Brain and CNS cancer	Pilocytic astrocytoma	15	↓	9.16×10^{-5}	–6.322	–8.917	35 (in top 1%)	Cancer Res 2002/04/01 (95)
	Glioblastoma	42	↓	3.80×10^{-4}	–5.350	–2.314	423 (in top 5%)	Oncogene 2003/07/31 (96)
	Anaplastic oligoastrocytoma	33	↓	0.004	–3.452	–1.634	1,053 (in top 6%)	Cancer Res 2006/12/15 (97)
Colorectal cancer	Rectal adenocarcinoma	105	↑	4.86×10^{-5}	7.292	1.703	500 (in top 3%)	Genome Biol 2007/07/05 (98)
	Rectosigmoid adenocarcinoma	105	↑	6.94×10^{-5}	5.439	1.653	1,158 (in top 6%)	Genome Biol 2007/07/05 (98)
	Colon mucinous adenocarcinoma	105	↑	6.08×10^{-5}	5.281	1.627	1,817 (in top 10%)	Genome Biol 2007/07/05 (98)
Kidney cancer	Chromophobe renal cell carcinoma	92	↑	4.49×10^{-17}	32.987	3.932	55 (in top 1%)	Clin Cancer Res 2005/08/15 (99)
	Renal oncocytoma	92	↑	2.49×10^{-21}	29.783	4.067	81 (in top 1%)	Clin Cancer Res 2005/08/15 (99)
	Chromophobe renal cell carcinoma	67	↑	4.54×10^{-4}	5.524	13.081	384 (in top 2%)	BMC Cancer 2009/05/18 (100)
	Clear cell sarcoma of the kidney	35	↑	0.002	3.783	2.287	703 (in top 6%)	Clin Cancer Res 2005/11/15 (101)
	Clear cell renal cell carcinoma	67	↓	0.002	–3.053	–1.743	1,314 (in top 7%)	BMC Cancer 2009/05/18 (100)
	Papillary renal cell carcinoma	67	↓	0.008	–2.592	–1.901	1,862 (in top 10%)	BMC Cancer 2009/05/18 (100)
Leukemia	Chronic lymphocytic leukemia	336	↑	2.22×10^{-5}	4.424	3.524	593 (in top 7%)	Nat Genet 2005/04/01 (102)
	Acute myeloid leukemia	293	↓	6.89×10^{-4}	–4.515	–1.676	313 (in top 3%)	N Engl J Med 2004/04/15 (103)
	T-cell prolymphocytic leukemia	14	↓	0.003	–3.494	–3.069	726 (in top 6%)	Leukemia 2007/10/01 (104)
Lung cancer	Lung carcinoid	203	↑	8.10×10^{-7}	6.593	6.774	120 (in top 2%)	Proc Natl Acad Sci U S A (105)
Lymphoma	Mantle cell lymphoma	336	↑	2.89×10^{-6}	6.249	5.514	182 (in top 3%)	Nat Genet 2005/04/01 (102)
Melanoma	Cutaneous melanoma	70	↑	2.94×10^{-8}	9.804	7.039	253 (in top 3%)	Clin Cancer Res 2005/10/15 (106)
Pancreatic cancer	Pancreatic ductal adenocarcinoma epithelia	25	↑	0.002	3.254	1.649	110 (in top 1%)	Neoplasia 2004/09/01 (107)
	Pancreatic ductal adenocarcinoma	38	↓	0.001	–4.137	–4.343	648 (in top 5%)	Oncogene 2005/10/06 (108)
Sarcoma	Clear cell sarcoma of the kidney	35	↑	0.002	3.783	2.287	703 (in top 6%)	Clin Cancer Res 2005/11/15 (101)
	Synovial sarcoma	54	↓	0.001	–3.655	–2.616	541 (in top 5%)	Cancer Res 2005/07/01 (109)
	Leiomyosarcoma	54	↓	0.004	–2.985	–2.238	957 (in top 8%)	Cancer Res 2005/07/01 (109)

Table S5 HCN3 expression in multiple types of cancer from NextBio Research database

Cancer types	Subtypes	P value	Fold change	N (sample)	References
Brain	Diffusely infiltrating astrocytic gliomas WHO grade II	0.0022	–1.7	5	(43)
	Pediatic tumor tissue pilocytic astrocytoma	0.0021	–1.8	15	(110)
	Pediatic tumor tissue ependymoma	0.0009	–1.9	14	(110)
	Glioblastoma multiforme tumors of neural subtype	3.10×10^{-9}	–2.2	N/A	(48)
	Pediatic tumor tissue diffuse astrocytoma	0.0149	–2.4	3	(110)
	Primary tumor dermal neurofibromas	0.0009	–2.5	13	(55)
	Pediatic tumor tissue glioblastoma	0.0014	–2.5	5	(110)
	Primary tumor malignant peripheral nerve sheath tumors	0.0005	–2.6	13	(55)
	Brain glioma stem cells	0.0011	–2.8	N/A	(41)
	Cultured NF1-derived primary benign neurofibroma Schwann cells	1.20×10^{-7}	–2.8	11	(56)
	Pediatic tumor tissue anaplastic astrocytoma	0.0029	–3.0	2	(110)
	Pediatic tumor tissue ependymoma	0.0006	–3.2	14	(110)
	Cultured plexiform neurofibroma-derived Schwann cell	1.10×10^{-7}	–3.5	11	(56)
	Primary tumor dermal neurofibroma	6.50×10^{-9}	–4.2	13	(56)
	Primary tumor plexiform neurofibroma	6.50×10^{-7}	–4.5	13	(56)
	Primary tumor malignant peripheral nerve sheath tumors	0.0005	–2.6	13	(55)
	Brain glioma stem cells	0.0011	–2.8	N/A	(41)
	Cultured NF1-derived primary benign neurofibroma Schwann cells	1.20×10^{-7}	–2.8	11	(56)
	Pediatic tumor tissue anaplastic astrocytoma	0.0029	–3.0	2	(110)
	Pediatic tumor tissue ependymoma	0.0006	–3.2	14	(110)
	Cultured plexiform neurofibroma-derived Schwann cell	1.10×10^{-7}	–3.5	11	(56)
	Primary tumor dermal neurofibroma	6.50×10^{-9}	–4.2	13	(56)
	Primary tumor plexiform neurofibroma	6.50×10^{-7}	–4.5	13	(56)
Breast	Primary breast tumor lobular carcinoma	0.0077	1.9	5	(111)
	Whole blood from patients with breast cancer	0.0132	1.7	11	(112)
	Whole blood from patients with breast cancer	0.0136	2.3	1	(112)
Leukemia	B lymphocytes (CD19+)—chronic lymphocytic leukemia	0.0004	2.2	47	GSE66117
Breast and lymphoma	Whole blood from patients with breast cancer and lymphoma	0.0302	1.6	2	(112)
Breast and intestinal	Whole blood from patients with breast and intestinal cancer	0.0367	1.9	1	(112)
Breast and gastric	Whole blood from patients with breast and gastric cancer	0.0345	1.9	1	(112)
Bladder	Primary resected bladder tumor	3.20×10^{-6}	1.9	165	(113)
	Bladder cancer tumors from TCGA patients	4.50×10^{-8}	2.9	223	TCGA
	Bladder cancer non-muscle invasive T1N0M0	0.0017	1.8	18	(114)
Colorectal	Normal epithelium from colorectal cancer patient	0.0011	2.6	8	(115)
Colon and ovarian	Whole blood from patients with colon and ovarian cancer	0.0039	1.8	5	(112)
Colon and lung	Whole blood from patients with colon and lung cancer	0.022	–2.1	1	(112)
Gastric	Gastric tumors biopsies	7.40×10^{-6}	–2.6	40	GSE33651
	Gastric cancer tissue	0.0089	1.5	8	(116)
Kidney	Renal tissue—pRCC1	1.90×10^{-11}	1.5	22	(77)
	Benign pheochromocytomas of adrenal glands SDHD mutant	0.0006	1.6	N/A	(117)
Liver	Hepatocellular carcinomas	3.60×10^{-5}	2.1	8	(118)
	Intrahepatic cholangiocarcinomas of proliferation subtype	0.0222	3.4	92	(60)
	Livers of hepatocellular carcinoma patients—non-recurrent tumor	4.50×10^{-11}	3.3	12	GSE56545
	Intrahepatic cholangiocarcinomas of inflammation subtype	0.0348	2.8	57	(60)
	Livers of hepatocellular carcinoma patients—recurrent tumor	6.30×10^{-8}	3.9	9	GSE56545
	Liver metastasis of midgut cancer	0.0298	1.8	3	(119)
	Hepatocellular carcinoma advanced stage tissue	0.0001	–3.4	13	(92)
	Hepatocellular carcinoma CTNNB1 mutant	0.0013	2.5	4	(120)
	Hepatocellular carcinoma early stage tissue	0.007	–1.8	13	(92)
Lung	Lungs frozen—carcinoid tumor	0.016	1.7	3	(121)
	Lung adenocarcinomas from TCGA patients	2.30×10^{-31}	3.6	454	TCGA
	Lung small cell carcinomas	5.90×10^{-8}	2.6	22	(122)
	Lungs in RNAlater—carcinoid tumor	0.0052	1.8	3	(121)
	Lung cancer cell line—SCLC	1.90×10^{-8}	2.0	22	(123)
	Lung carcinoid tumors	6.50×10^{-13}	3.1	24	(122)
Melanoma	Melanoma skin biopsies—primary melanoma	8.90×10^{-5}	–2.7	46	(53)
	Primary melanoma—superficial spreading melanoma	0.0003	–2.5	28	(53)
	Metastatic melanoma in short term culture	0.0231	–2.3	1	(124)
Ovarian	Blood fraction of patients with epithelial ovarian cancer	8.60×10^{-5}	–1.9	48	GSE31682
Ovarian and bladder	Whole blood from patients with ovarian and bladder cancer	0.048	1.8	1	(112)
Prostate	Metastatic prostate tumor samples	0.0005	2.6	25	(125)
Sarcoma	Pleomorphic liposarcoma tissues	0.0043	1.6	8	(126)

pRCC1, type 1 papillary renal cell carcinoma.

Table S6 HCN3 expression in multiple types of cancer from Oncomine database

Cancer	Subtype	N (case)	Expression	P value (cancer/normal)	*t*-test (cancer/normal)	Fold (cancer/normal)	Gene ranking (%)	Database
Breast cancer	Intraductal cribriform breast adenocarcinoma	593	↑	0.005	6.153	1.646	1,856 (in top 10%)	TCGA Breast 2011/09/02
Prostate cancer	Prostate carcinoma	122	↓	6.02×10^{-5}	–4.257	–1.560	1,864 (in top 10%)	Nature 2012/05/20 (127)

Table S7 HCN4 expression in multiple types and subtype of cancer from NextBio Research database

Cancer subtypes	P value	Fold change	N (sample)	References
Thyroid carcinomas from TCGA patients vs. normal thyroid tissue	9.00×10^{-58}	30.36033183	500	TCGA
Livers of patients with intrahepatic cholangiocarcinoma—tumor vs. surrounding non-tumorous tissue	4.10×10^{-7}	11.20081464	7	(128)
Thyroid tissues—papillary thyroid carcinoma patients vs. healthy donors	5.40×10^{-9}	10.02491379	18	(72)
Primary tumor of midgut cancer vs. ileum enriched for NE producing cells	0.0483	8.27	3	(119)
Primary tumor of midgut cancer vs. normal ileum	0.0492	8.26	3	(119)
Whole blood from patients with ovarian and bladder cancer vs. healthy	0.0036	5.06	1	(112)
Pediatric cerebellum—from medulloblastoma patients (aged 27–51 months) vs. normal	0.0044	4.18	8	(51)
Lungs frozen—carcinoid tumor vs. normal controls	0.0398	4.17	3	(121)
Bladder cancer tumors from TCGA patients vs. normal bladder tissue	0.0175	3.879884125	223	TCGA
Lung carcinoid tumors vs. non-tumor controls	7.50×10^{-12}	3.79	24	GSE30219
Lungs in RNAlater—carcinoid tumor vs. normal controls	0.0417	3.76	3	(121)
Breast cancer tumors from TGCA patients vs. normal breast tissue	1.70×10^{-9}	3.233176205	1,018	TCGA
Liver hepatocellular carcinomas from TCGA patients vs. normal liver tissue	0.0017	3.026373943	165	TCGA
Clear cell sarcoma of kidney vs. fetal kidney	0.0061	2.61	3	(101)
Molecular profiling of PBMC from T-cell LGL vs. pooled control	0.0052	1.65	30	(91)
Prostate cancer stage T2 vs. normal	0.0227	1.63	3	(82)
Esophageal biopsies—cancerous tissue vs. normal tissue GPL8300	2.60×10^{-7}	1.57	15	(129)
Tubulointerstitium of tumor nephrectomy vs. normal cadaver controls GPL14663	0.0033	–1.5	11	(129)
Thyroid tumors vs. unmatched normal thyroid biopsies	0.0095	–1.52	19	(130)
Bone marrow CD34+ progenitor cells from myelodysplastic syndrome patients vs. normal	0.0496	–1.63	14	(131)
PBMC of patients with gastrointestinal or brain cancer vs. normal mammogram	1.50×10^{-7}	–1.65	22	(132)
Bone marrow from acute lymphoblastic leukemia vs. thymocytes CD8+ single positive	7.00×10^{-5}	–1.69	31	GSE46170
Differentiated hepatocellular carcinoma grade III–IV vs. normal liver	7.50×10^{-5}	–1.85	12	GSE36411
HCC with fibrous stroma vs. normal surrounding liver tissue	0.0227	–1.89	7	(133)
Plasma cells from multiple myeloma patients vs. healthy bone marrow GPL8300	0.0168	–2.07	12	GSE9656
Bone marrow plasma cells—monogammopathy of uncertain significance vs. normal	0.0002	–2.12	44	(134)
Undifferentiated hepatocellular carcinoma grade III–IV vs. normal liver	0.0002	–2.14	8	GSE36411
Esophagus tumors vs. adjacent matched normal esophagus biopsies	0.0004	–2.28	13	(130)
Liver cholangiocarcinoma vs. normal surrounding liver tissue	0.0064	–2.54	6	(133)
Malignant pleural mesothelioma tumors vs. normal pleura	0.0173	–2.91	40	(135)
Prostate adenocarcinomas from TCGA patients vs. normal prostate tissue	8.50×10^{-23}	–3.691279781	259	TCGA
Gastric tissue—primary gastric tumor vs. normal tissue	0.0192	–5.50412551	3	GSE41476
Stage II endometrial carcinoma vs. normal endometrial epithelium	0.0147	–135	72	(136)
Stage I endometrial carcinoma vs. normal endometrial epithelium	0.0136	–157	72	(136)

HCC, hepatocellular carcinoma.

Table S8 HCN4 expression in multiple type and subtype of cancer from Oncomine database

Cancer type	Subtype	N (case)	Expression	P value (cancer/normal)	*t*-test (cancer/normal)	Fold (cancer/normal)	Gene ranking (%)	Database
Bladder cancer	Infiltrating bladder urothelial carcinoma	60	↓	2.27×10^{-5}	–5.259	–1.557	1,060 (in top 9%)	Cancer Res 2004/06/01 (137)
Breast cancer	Invasive ductal breast carcinoma	63	↓	1.66×10^{-4}	–4.426	–2.551	146 (in top 1%)	Proc Natl Acad Sci U S A 2005/08/02 (138)
Esophageal cancer	Barrett's esophagus	24	↓	4.81×10^{-4}	–4.929	–2.899	91 (in top 1%)	Cancer Res 2005/04/ (139)
Kidney cancer	Clear cell sarcoma of the kidney	35	↑	1.26×10^{-4}	5.505	3.007	338 (in top 3%)	Clin Cancer Res 2005/11/15 (101)
	Renal pelvis urothelial carcinoma	92	↓	2.99×10^{-22}	–29.961	–3.660	4 (in top 1%)	Clin Cancer Res 2005/08/15 (99)
	Chromophobe renal cell carcinoma	92	↓	8.32×10^{-5}	–6.343	–1.552	1,068 (in top 9%)	Clin Cancer Res 2005/08/15 (99)
Prostate cancer	Prostate carcinoma	30	↑	0.005	2.788	7.887	291 (in top 2%)	Mol Carcinog 2002/01/01 (108)
Sarcoma	Clear cell sarcoma of the kidney	35	↑	1.26×10^{-4}	5.505	3.007	338 (in top 3%)	Clin Cancer Res 2005/11/15 (101)

Table S9 Preferred reporting items for systematic review and meta-analysis protocols checklist (140)

Section/topic	#	Checklist item	Reported on page
Title			
Title	1	Identify the report as a systematic review, meta-analysis, or both	1
Abstract			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number	1
Introduction			
Rationale	3	Describe the rationale for the review in the context of what is already known	1
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS)	2
Methods			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number	2
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale	2
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched	
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated	2
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis)	2
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators	2
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made	2
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis	2
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means)	2
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis	2
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies)	2
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified	3,4
Results			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram	4
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations	4
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12)	4
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (I) simple summary data for each intervention group; (II) effect estimates and confidence intervals, ideally with a forest plot	5–8
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency	5–8
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see item 15)	5–8
Additional analysis	23	Give results of additional analyses, if done [e.g., sensitivity or subgroup analyses, meta-regression (see item 16)]	5–8
Discussion			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers)	9
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias)	10
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research	10
Funding			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review	10

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