

Original Article

Correlation between BOLD-MRI and HIF expression level in renal carcinoma

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Abstract: Occupying about 2%~3% of all malignant tumors, renal carcinoma is the most common primary cancer in kidney. The oxidative level of tumor cells is of vital role for optimizing treatment plan, evaluating efficacy and predicting prognosis. This study thus investigated the $R2^*$ value in mouse renal carcinoma model and the correlation between tumor hypoxia and expression level of hypoxia inducible factor-1 (HIF-1). A total of 20 BALB/C nude mice (4~6 weeks old) were inoculated with human ACHN renal carcinoma cells to generate renal cancer model. After the tumor diameter reached 0.5 cm, all animals were examined by BOLD-MRI, both under normal inhalation ($R2a^*$) and carbogen treatment ($R2b^*$). The alternation of $R2^*$ values ($\Delta R2^* = R2a^* - R2b^*$) was calculated. Mice were then sacrificed for Immunohistochemical (IHC) staining targeting HIF-1 α and HIF-2 α . The positive score of HIF was then analyzed for its correlation with $R2^*$ value. In 18 mice finished both experiments, Pearson correlation analysis revealed significant negative correlation between $R2a^*$ and $\Delta R2^*$ ($r = -0.48$, $P < 0.05$) and positive relationship between $\Delta R2^*$ and HIF-2 α ($r = 0.38$, $P < 0.05$). HIF-1 α level, however, did not correlated with tumor R^* values. The positive correlation between $\Delta R2^*$ and HIF-2 α , but not HIF-1 α , suggested potential role of combined BOLD-MRI technique and HIF-1 α staining in clinical diagnosis of renal carcinoma. HIF-2 α may work as biological marker for renal cancer.

Keywords: Renal carcinoma, BOLD-MIR, hypoxia inducible factor, correlation analysis

Introduction

Renal cancer consists almost 85%~90% of all primary malignant renal tumors and occupies about 2%~3% of all cancers [1]. In each year, there are about 209,000 newly discovered cases of renal carcinoma worldwide [2]. In different regions of China, the overall incidence and mortality of renal cancer is growing, although the existence of regional difference [3].

Hypoxia inducible factor (HIF) exerts important roles in the pathogenesis of multiple tumors. It is firstly identified from nuclear extracts of hypoxia-induced cells [4], and is one of major transcriptional factors regulating gene expression under hypoxia condition. It is widely distributed in human cells and is composed of α - and β -subunits. Hypoxia and HIF-1 are known to be related with various tumor progressive factors. For example, the blocking of hypoxia pathway can lead to metabolic disorder, depressed

angiogenesis, and even programmed death of tumor cells. There have been various experiments to inhibit HIF-1 at transcriptional, translational and intracellular activity levels, to facilitate the hypoxia treatment of cancer. The accurate monitor of oxidative level of tumor cells, is thus of critical importance for optimizing treatment strategy, evaluating efficacy and predicting prognosis. Cytoplasmic HIF-1 α level has been known to be up-regulated in renal carcinoma with higher malignancy [5]. Both HIF-1 α and HIF-2 α are shown be abundantly expressed in clear cell renal cell carcinoma (CRCC), along with the correlation between HIF- α positive rate and pathological grade of CRCC [6, 7]. Therefore, HIF- α plays a crucial role in both occurrence and progression of CRCC.

Functional magnetic resonance imaging (fMRI) of renal functions is a novel non-invasive technique to evaluate renal functions. It can provide information regarding both kidney morphology and kidney functions on routine MRI equipment

without using contrasts. It has been widely applied in various kidney diseases including chronic renal failure, kidney transplantation, hypertensive kidney dysfunction, ischemic renal injury and renal tumors [8, 9]. One novel fMRI technology, blood oxygenation level-dependent MRI (BOLD-MRI) can reflect the oxygen pressure inside tissues, via the magnetic signals variations as the result of hemoglobin/oxygenated hemoglobin. This technique is sensitive in evaluating the acute hypoxia condition inside tissues [10]. This study thus investigated the correlation between $R2^*$ values obtained from BOLD-MRI on hypoxic tumor tissues and HIF, which can work as one endogenous marker for tumor hypoxia, in an attempt to provide evidences for optimizing clinical treatment of kidney cancer.

Materials and methods

Animal model

A total of 20 BALB/C nude mice (both males and females, aging between 4~6 weeks, body weight: 15~20 g) were kept in a specific pathogen free (SPF) grade animal facility with food and water *ad libitum*. Mice were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Liyang people's Hospital.

Human ACHN renal carcinoma cells (Aiyuan Bio., Shanghai, China) were cultured in DMEM with changing every 48 hours. Cells were digested by 0.25% trypsin and rinsed in D-Hank solution. After centrifugation and discarding supernatants, cells were re-suspended in serum-free medium at 1.0×10^7 per mL.

Mice were anesthetized by 1% pentobarbital (0.15 mL) via intraperitoneal injection. After skin sterilization, 0.2 mL ACHN cell suspensions were injected subcutaneously into the forelimb axillary skin.

BOLD-MRI

All mice were examined under BOLD-MRI (Achieva 3.0T, GE, US) using phased array coils for small animals (5 cm diameter). Examinations were performed before and after carbogen inhalation. Computer software was used to analyze all captured images. $R2^*$ values were averaged across all planes fitted imaging crite-

ria. $R2^*$ values during air inhalation were marked as $R2a^*$, while those values captured 10 min after carbogen inhalation were designed as $R2b^*$. $\Delta R2^*$ was calculated as $R2b^* - R2a^*$.

Immunohistochemical (IHC) staining

Mice after BOLD-MRI were then sacrificed. Renal samples were firstly fixed in 10% neutral buffered formalin (NBF), followed by paraffin embedding and sectioning (4 μ m thickness). After de-wax and re-hydration, tissues slides were quenched for hydroxylase activity, blocked by non-immune serum, and was incubated with mouse anti-human HIF-1 α and HIF-2 α monoclonal antibody (Yixin Biotech., Shanghai, China). Secondary antibody and streptomycin-conjugated anti-biotin and peroxidase were sequentially applied to develop the slides using DAB substrates. After counter-staining in hematoxylin, images were captured under high magnification ($\times 400$) light-field microscope (Olympus, Japan). In each tissue section, 3 fields were randomly selected for counting 200 cells in each field. The positive cell number was counted and overall positive scores were given as 0 (less than 1% of positive cells), 1 (1%~10% positive cells), 2 (11%~50% positive cells), 3 (51%~80% positive cells) and 4 (more than 80% positive cells). Moreover, staining strength score was also given as 0 (no staining), 1 (light yellow), 2 (dark yellow) or 3 (dark brown). The final score of staining was given by the summation of positive score and strength score.

Statistical analysis

SPSS 18.0 software was used to process all collected data, of which measurement data were presented as mean \pm standard deviation (SD). All data were firstly tested for normal distribution using one-sample K-S test. Pearson analysis was used to examine the correlation between parameters. A statistical significance was defined when $P < 0.05$.

Results

$R2a^$ and $\Delta R2^*$ values in renal carcinoma tissues*

Among all 20 mice, 2 of them died from anesthesia, and the remaining 18 mice completed all MRI examination and IHC staining (N=18). We found 16 mice had depressed $R2^*$ values

Table 1. R2a* and ΔR2* values and normality test (K-S test, N=18)

	R2a* (S ⁻¹)	ΔR2* (S ⁻¹)
Mean ± SD	38.13±14.32	-2.35±2.07
Z	0.91	0.71
P	0.32	0.62

Table 2. HIF-1α and HIF-2α staining score and normality test (K-S test, N=18)

	HIF-1α	HIF-2α
Mean ± SD	3.8±1.25	2.91±1.49
Z	0.74	0.55
P	0.64	0.85

Table 3. Correlation analysis of HIF-1α/2α expression and R2a* and ΔR2*

	ΔR2*		HIF-1α		HIF-2α	
	r	P	r	P	r	P
R2a*	-0.48	0.0032	0.04	0.79	0.47	0.0029
ΔR2*			0.27	0.17	0.38	0.0044

after carbogen inhalation, while 2 of them had increase levels (**Table 1**), suggesting individual differentiation in the response to carbogen.

HIF-1α and HIF-2α expression

Both HIF-1α and HIF-2α were specifically expressed in the nucleus. These two factors were shown to diffuse within tumor tissues or in the adjacent sites (**Table 2**).

Correlation between HIF-1α/2α expression and R2a and ΔR2* values*

Using one-sample K-S test, the distribution of HIF-1α, HIF-2α, R2a* and ΔR2* values all fitted the normal distribution. Pearson correlation analysis showed significantly negative correlation between R2a* and ΔR2* values (r=-0.48, P<0.05), while both R2a* or ΔR2* values were positively correlated with HIF-2α (r=0.47 and 0.38, P<0.05, **Table 3**).

Discussion

HIF is one of major transcriptional factors regulating gene expression under hypoxia condition and is widely distributed in human cells. Composing of α- and β-subunits, HIF family members share certain structural similarities.

The α-subunit, including HIF-1α, HIF-2α and HIF-3α, exert unique inducing mechanisms due to differential expression. After dimerization between HIF-α and HIF-β, dozens of hypoxia responsive genes (HRGs) can be activated. HIF-1 and HIF-2 are known to participate in the pathological process for tissue hypoxemia. Due to the uncontrolled growth, tumor tissues may suffer from hypoxia, causing increased stability of intracellular HIF-α, which can form dimers with β-subunits. Such actions may lead to abnormal down-stream gene expression, such as the vascular endothelial growth factor (VEGF) and angiogenesis. HIF-1α is widely distributed in various tumors, with its expression level increased by the tissue hypoxia condition. The accumulation of HIF-1α thus may works as one endogenously index for *in vivo* assay of hypoxia [11].

Studies have revealed the absence of HIF-1α in normal kidney tissues but significantly elevation in CRCC [12] rather than other types of renal cancer. The reason for up-regulation of HIF-1α in renal carcinoma tissues may be due to the hypoxia condition under renal cancer. A clinical survey showed 37.5% of non-CRCC patients had HIF-1α up-regulation. This figure in CRCC, however, was 75% [13]. No matter under hypoxia or normal condition, HIF-2α exerts a more powerful role in transcriptional activation of VEGF mRNA compared to HIF-1α, suggesting more important role of HIF-2α signaling pathway in the occurrence and progression of renal carcinoma [14, 15]. Previous study has revealed the potentiation of HIF-2α expression in CRCC, along with a positive correlation between HIF-2α level and tumor invasiveness [12]. The up-regulation of HIF-2α mainly existed in CRCC as proved by previous study [16]. In summary, both HIF-1α and HIF-2α play an important role in both occurrence and progression of renal carcinoma, and may work as predicative indexes for tumor metastasis and invasion. In this study we also found positive expression of HIF-1α and HIF-2α in renal carcinoma tissues, as agreed with previous findings.

BOLD-MRI uses the influence of hemoglobin/oxygenated hemoglobin on magnetic signal strength, thus indirectly predicting the distribution of oxygen inside tissues. This is a high-sensitive assay for examining acute tissue hypoxia [10]. The R2* value of BOLD-MRI was known to be positively correlated with tissue contents of

hemoglobin. Previous findings reported the elevated oxygen pressure after carbogen inhalation using both BOLD-MRI and microelectrode assays on mouse squamous carcinoma cells [17]. Further study supported the correlation between $R2^*$ value of BOLD-MRI and tissue hypoxia score (HP5) and oxygen pressure, in addition to the tumor distribution of HIF-1 [18]. A recent *in vivo* study suggested decreased HIF-1 α expression and lowered lactate byproducts in tumor cells after exogenously application of HIF inhibitor [19]. Both HIF- α and $R2^*$ value are correlated with tissue oxygen contents. Studies cannot identify significant correlation between HIF- α expression level and $R2^*$ value in either high malignancy or low malignancy group. However, HIF-2 α expression rate was shown to be positively correlated with $R2^*$ value in CRCC with different TNM stages [20, 21]. Our study revealed negative relationship between $R2a^*$ and $\Delta R2^*$ values, while both $R2a^*$ or $\Delta R2^*$ values were positively correlated with HIF-2 α . In summary, our results suggested the potential role of BOLD-MRI in clinical treatment of renal carcinoma, when combining with HIF-2 α expressional assay. This study provided further evidences regarding the potency of HIF- α in an independent or combined molecular marker for renal cancer.

Disclosure of conflict of interest

None.

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