Over-expression of \textit{BCAT1}, a c-Myc target gene, induces cell proliferation, migration and invasion in nasopharyngeal carcinoma

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Abstract

\textbf{Background:} Nasopharyngeal carcinoma (NPC) is a common malignant tumor in southern China and Southeast Asia, but its molecular mechanisms of pathogenesis are poorly understood. Our previous work has demonstrated that \textit{BCAT1} mRNA is over expressed in NPC and knocking down its expression in 5-8F NPC cell line can potently inhibit cell cycle progression and cell proliferation. However, the mechanism of \textit{BCAT1} up-regulation and its functional role in NPC development remain to be elucidated yet.

\textbf{Methods:} Immunohistochemistry (IHC) method was utilized to detect the expression of BCAT1 protein in NPC at different pathological stages. The roles of gene mutation, DNA amplification and transcription factor c-Myc in regulating \textit{BCAT1} expression were analyzed using PCR-sequencing, quantitative polymerase chain reaction (qPCR), IHC, ChIP and luciferase reporter system, respectively. The functions of \textit{BCAT1} in colony formation, cell migration and invasion properties were evaluated by RNA interference (RNAi).

\textbf{Results:} The positive rates of BCAT1 protein expression in normal epithelia, low-to-moderate grade atypical hyperplasia tissues, high-grade atypical hyperplasia tissues and NPC tissues were 23.6\% (17/72), 75\% (18/24), 88.9\% (8/9) and 88.8\% (71/80), respectively. Only one SNP site in exon1 was detected, and 42.4\% (12/28) of the NPC tissues displayed the amplification of microsatellite loci in \textit{BCAT1}. C-Myc could directly bind to the c-Myc binding site in promoter region of \textit{BCAT1} and up-regulate its expression. The mRNA and protein of c-Myc and \textit{BCAT1} were co-expressed in 53.6\% (15/28) and 59.1\% (13/22) of NPC tissues, respectively, and \textit{BCAT1} mRNA expression was also down-regulated in c-Myc knockdown cell lines. In addition, \textit{BCAT1} knockdown cells demonstrated reduced proliferation and decreased cell migration and invasion abilities.

\textbf{Conclusions:} Our study indicates that gene amplification and c-Myc up-regulation are responsible for \textit{BCAT1} overexpression in primary NPC, and overexpression of \textit{BCAT1} induces cell proliferation, migration and invasion. The results suggest that \textit{BCAT1} may be a novel molecular target for the diagnosis and treatment of NPC.

\textbf{Keywords:} Nasopharyngeal carcinoma, \textit{BCAT1}, c-Myc, Proliferation, Migration, Invasion, Gene amplification, Gene regulation
Background

Nasopharyngeal carcinoma (NPC) is a squamous cell carcinoma that develops from the epithelium of the nasopharynx with a high incidence in Southeast Asia and southern China and causes a serious healthcare problem in these regions [1]. More than 95% of NPC in southern China is undifferentiated carcinoma with a high incidence of early metastasis which is the main cause of death in NPC patients. Currently, radiation therapy is the first choice for NPC treatment. Although the radiotherapy equipments and techniques have been improved tremendously, the five-year survival rate of NPC patients has not radically changed yet and remains around 50-60%. Therefore, it is of great importance to comprehensively explore the new approaches for NPC treatment.

The molecular mechanisms of nasopharyngeal carcinogenesis have not been elucidated clearly yet. Previous studies have shown that proto-oncogenes (e.g., HRAS, NRAS2 [2], cyclin D1 [3], MDM2 [4], EVI1 [5], EGFR [6]) and tumor suppressor genes (TSGs) (e.g., p53 [7], p16 [8], RASSF1A [9], DLC-1 [10], LTIF [11,12], DLEC1 [13], TSLC1 [14]) are aberrantly expressed in NPC. Using comparative genomic hybridization (CGH) data from 170 primary NPC cases, we have developed a tree model indicating the pathogenetic mechanisms of NPC [15]. According to the tree model, +12p11-12 may represent an early event in the carcinogenesis of NPC [15].

We further identified a microsatellite locus at $12p11-12$ that may represent an early event in NPC pathogenesis. Since gene mutation and DNA amplification are two major causes for oncogene up-regulation, we first performed DNA sequencing of the full-length of 11 exons in $BCAT1$. Only one polymorphism (G/T) was detected at +78 in the non-coding region of first exon (Figure 2A), which was further confirmed in the single nucleotide polymorphism (SNP) database.

Results

The expression of $BCAT1$ protein increased significantly at early stage of NPC

To evaluate the significance of $BCAT1$ in NPC pathogenesis, we investigated the expression of $BCAT1$ protein in different stages of precancerous and cancerous lesions in nasopharyngeal biopsies. Cytoplasmic immunostaining signals of $BCAT1$ could be detected at different stages, but the positive rates differed greatly, which were 23.6% (17/72), 75.0% (18/24), 88.9% (8/9) and 88.8% (71/80) in normal epithelia, low-to-moderate grade atypical hyperplasia tissues, high-grade atypical hyperplasia tissues and NPC tissues, respectively (Figure 1, Table 1, $P < 0.05$), indicating that up-regulation of $BCAT1$ is an early event in NPC pathogenesis.

No mutation of $BCAT1$ was found in NPC tissues

Since gene mutation and DNA amplification are two major causes for oncogene up-regulation, we first performed DNA sequencing of the full-length of 11 exons in $BCAT1$. Only one polymorphism (G/T) was detected at +78 in the non-coding region of first exon (Figure 2A), which was further confirmed in the single nucleotide polymorphism (SNP) database.

Frequent amplification of $BCAT1$ was detected in NPC tissues

Three microsatellites (D12S1435, D12S1617 and RH44650) located within $BCAT1$ gene were selected for analysis of $BCAT1$ amplification. Real-time PCR was employed to detect DNA samples from 28 NPC tissues and their matched peripheral blood specimens. The amplification ratios of D12S1435, D12S1617 and RH44650 were 14% (4/28), 25% (7/28) and 17% (5/28), respectively (Figure 2B). The total amplification ratio was 42.4% (12/28).

The transcription factor c-Myc regulated $BCAT1$ expression

By searching NNPP and TESS, a c-Myc recognition site (CACGTG) was discovered in the 5’ regulatory region of $BCAT1$ gene, suggesting that expression of $BCAT1$ may be regulated by the transcription factor c-Myc. ChIP experiment using anti-c-Myc antibody was carried out to co-precipitate DNA sequences binding to c-Myc. The specific primers at $-233$ to $-41$ bp of $BCAT1$ were designed. As shown in Figure 3A, a 193 bp fragment of $BCAT1$ sequence was amplified, indicating that c-Myc transcription factor can directly bind to the specific promoter region of $BCAT1$ gene.

Subsequently, we analyzed the regulation of $BCAT1$ by c-Myc through knocking down c-Myc expression in NPC cells. When c-Myc shRNA vectors were transfected into 5-8F and 6-10B NPC cells, the mRNA expression of c-Myc decreased by 80% and 70% in 5-8F-Si-c-Myc and 6-10B-Si-c-Myc cells, respectively, as measured by semi-quantitative RT-PCR. As expected, the expression of $BCAT1$ was also inhibited by 85% and 72% in 5-8F-Si-c-Myc and 6-10B-Si-c-Myc cells, respectively. Meanwhile, the expression of $KRAS$ and $MCAM$, two c-Myc
non-target genes, was unaffected by c-Myc knockdown (Figure 3B), further supporting that c-Myc can regulate BCAT1 expression.

To further test whether c-Myc regulates BCAT1 expression, we performed luciferase assay. The COS7 cells with absent expression of c-Myc were co-transfected with pGL3-233/-41 recombinant and c-Myc expression vector. The results showed that the luciferase activity of reporter system in co-transfected cells was markedly higher than that in parental COS7 cells and COS7 cells transfected with pGL3-233/-41-M mutant in which the c-Myc binding site was mutated (Figure 3C). We also conducted the luciferase assay in 5-8F-Si-c-Myc cells. Similarly, once c-Myc was knocked down in 5-8F cells, luciferase activity of pGL3-233/-41 recombinant dramatically decreased (Figure 3C), but that of pGL3-233/-41-M mutant had no significant change. Together, these results indicate that c-Myc directly binds to promoter of BCAT1 and transactivates its expression.

Expression of c-Myc and BCAT1 was detected in NPC tissues

The mRNA expression of c-Myc and BCAT1 was detected by RT-PCR in 6 chronic nasopharyngitis (CN) samples and 28 NPC samples. The results showed that c-Myc and BCAT1 mRNA expression were low or undetectable in 6 CN tissues, while over expression of c-Myc and BCAT1 was found in 67.9% (19/28) and 64.3% of the NPC tissues.

| Groups                                    | No. | Negative No.(%) | Positive No.(%) |
|-------------------------------------------|-----|----------------|----------------|
| Normal epithelia                          | 72  | 42(58.3)       | 13(18.1)       |
| Low-to-moderate grade atypical hyperplasia | 24  | 5(20.8)        | 1(4.2)         |
| High-grade atypical hyperplasia tissues   | 9   | 1(11.1)        | 0              |
| NPC tissues                               | 80  | 7(8.7)         | 2(2.5)         |

*P* value was calculated by comparing the positive rate of BCAT1 in low-to-moderate grade atypical hyperplasia tissues, high-grade atypical hyperplasia tissues and NPC tissues with that in normal epithelia, respectively.
(18/28) of NPC tissues, respectively. In addition, c-Myc and BCAT1 exhibited the same mRNA expression patterns in 74% of NPC tissues, as they were lowly expressed in 21% (6/28) and co-upregulated in 53% (15/28) of NPC tissues (Figure 3D, Table 2).

The protein expression of c-Myc and BCAT1 was also examined by IHC in 22 NPC samples. The c-Myc or BCAT1 protein was positively stained in 73% (16/22) and 68% (15/22) of NPC tissues, respectively. Among them, c-Myc and BCAT1 were simultaneously and positively stained in 59% (13/22), whereas lowly or negatively in 18% (4/22) of NPC tissues (Figure 3E, Table 2).

The results showed a positive correlation of c-Myc expression and BCAT1 expression in NPC tissues (Table 2, \(P = 0.019\) for RT-PCR; \(P = 0.032\) for IHC).

**Discussion**

CGH-array is a newly developed technique for detecting genetic lesions in cancer and other diseases [18]. Numerous genetic abnormalities have been identified in multiple chromosomal regions in NPC tissues and cell lines [19]. Frequent gains on 1q, 3q, 8q, 11q, 12p and 12q, and losses on 3p, 9p, 11q, 14q and 16q, have been found. Moreover, several minimal regions of gains including 3q27.3-28, 8q21-24 and 11q13.1-13.3 have been identified and several minimal deleted regions have been mapped to 3p14.1-22, 11q13.3-24, 13q14.3-22, 14q24.3-32.1 and 16q22-23 [19]. We have analyzed 170 comparative genomic hybridization (CGH) samples and constructed a tree model to predict NPC tumorigenesis. We are particularly interested in the gain of 12p11-12 (+12p11-12) since +12p11-12 is a region frequently amplified and may be an early event in the development of NPC [15].

**BCAT1** is located at 12p12.1, and codes for the cytosolic form of branched-chain amino acid transaminase which catalyzes the reversible transamination of branched-chain alpha-keto acids to branched-chain L-amino acids essential for cell growth. BCAT1 has been reported to be highly conserved in evolution and disruption of its yeast homolog affects cell growth [20,21]. Several groups have confirmed that **BCAT1** is involved in cell proliferation, cell cycle progression, differentiation and apoptosis, and plays an important role in several malignancies, especially in the progression of nonseminomas [22-24]. The mouse homologue of **BCAT1** has been shown to be amplified and overexpressed in a teratocarcinoma cell line [25]. Retroviral transduction of **BCAT1** into fetal rat brain cells with SV40 large T-antigen induced tumor formation with characteristic features of medulloblastoma [26].
Previously, RT-PCR results have presented that BCAT1 is significantly up-regulated in NPC tissues and silencing its expression blocks NPC cell proliferation and the G1/S transition, indicating that high expression of BCAT1 may play an important role in NPC cell survival [16].

Here, we further performed IHC analysis of different stages of NPC and found that BCAT1 protein level increased in the low-to-moderate grade atypical hyperplasia tissues as well as high-grade atypical hyperplasia tissues, in situ and invasive carcinomas, suggesting that...
BCAT1 overexpression may be an important early event in NPC occurrence and maintain throughout NPC progression. There are several factors that can account for the abnormalities of gene expression, such as gene mutation, DNA amplification, transcriptional regulation and epigenetic changes, alone or synergistically. Gene mutation and amplification are two common causes for genetic activation of oncogenes. It is well known that Ras mutation is closely related to various malignancies such as breast cancer and lung cancer [27], and TRK mutation is also found to be associated with neuroblastoma [28]. HER-2/neu amplification is frequently detected in node-negative breast carcinoma tissues and it is a good example for oncogene activation by gene amplification [29]. We first analyzed whether BCAT1 has mutation by sequencing 11 exons of BCAT1 in 20 cases of NPC. Only one SNP site in exon1 was detected, suggesting that gene mutation of BCAT1 is a rare incident in NPC. By using real-time PCR, we also analyzed three microsatellite loci including D12S1435, D12S1617 and RH44650 to examine whether BCAT1 is amplified in NPC. Our results demonstrated that 42.4% (12/28) of NPC tissues manifested amplification, revealing that BCAT1 overexpression may be due to its amplification in a portion of NPC tissues. Genes such as CDH13 [30], p16 and p27 [8] have been reported to be involved in the early development of NPC. BCAT1 over-expression, together with abnormal expression of CDH13, p16, p27 and others, may result in transition from normal epithelia to hyperplastic epithelia.

BCAT1 was first identified from a c-Myc-induced tumor and has been proven to be directly regulated by c-Myc through its binding to the specific DNA sequence, CACGTG [17,25]. C-Myc is an oncogene and transcription factor involved in the tumorigenesis of multiple cancers, such as Burkitt’s lymphoma and breast cancer [17]. Both BCAT1 and c-Myc were found to be

### Table 2 Correlation analysis between c-Myc and BCAT1 expression in the same batch of NPC tissues

| BCAT1 | c-Myc | RT-PCR | IHC |
|-------|-------|--------|-----|
|       |       | L | U | Total | L | U | Total |
| L     | 6     | 4 | 10 | 4 | 3 | 7 |
| U     | 3     | 15 | 18 | 2 | 13 | 15 |
| Total | 9     | 19 | 28 | 6 | 16 | 22 |

P = 0.019  P = 0.032

U up-expression, L low expression.

Figure 4 Detection of the colony formation ability, migration and invasion capacities of NPC cells. The colony formation ability (A), migration (B) and invasion capacities (C) of 5-8F cells decreased when the expression of BCAT1 was blocked.
overexpressed in NPC [31]. We thus used IHC, RNAi, ChIP and Luciferase reporter system to investigate whether BCAT1 is directly regulated by c-Myc in NPC. 59% of NPC tissues were double positive for c-Myc and BCAT1. Silencing the endogenous expression of c-Myc by RNAi also decreased BCAT1 mRNA level in 5-8F-Si-c-Myc and 6-10B-Si-c-Myc cells. Using luciferase assay, we found transcription factor c-Myc up-regulated BCAT1 expression. Furthermore, we confirmed that c-Myc can directly bind to the BCAT1 promoter. Our results revealed that c-Myc, together with BCAT1 amplification, up-regulates BCAT1 expression and leads to BCAT1 activation in NPC tissues.

One of the major clinical features of NPC is early metastasis. Several genes have been found to be associated with the metastasis of NPC, for example, LMP1, LMP2A, p16, nm-23, CD44v6, TSLC1, NGX6, MMP9 and LTF [6,9,32-35]. However, they cannot fully elucidate the mechanisms underlying NPC metastasis. In this study, we indicated increased expression of BCAT1 in the premalignant and NPC tissues. By performing colony formation, migration and invasion assays, we showed that colony formation, cell mobility and invasion abilities of 5-8F cells were reduced in response to knockdown of BCAT1 expression. Consistent with our data, high BCAT1 expression is associated with a high incidence of metastasis resulting in an adverse disease-free survival in colorectal adenocarcinomas [36]. Both mRNA and protein levels of BCAT1 are higher in medulloblastoma patients with metastasis compared with those without metastasis (\( P < 0.01 \)) [37]. Taken together, BCAT1 may be a favorable biomarker to indicate NPC early metastasis.

Conclusion
In summary, for the first time, we demonstrate that expression of BCAT1, which locates in the frequently amplified 12p12 region, increases at early pathological stage of NPC. Gene amplification is an important cause for overexpression of BCAT1 in NPC, while c-Myc also plays a critical role in regulation of BCAT1 expression. We also confirm that high expression of BCAT1 is associated with the mobility of NPC cells, indicating that it may be a promising target for NPC diagnosis and treatment.

Methods
Cell culture
5-8F, 6-10B and COS7 cells were cultured in RPMI1640 (Gibco BRL, Bethesda, MD) media with 10% fetal bovine serum (FBS) at 37°C in an atmosphere containing 5% CO\(_2\). The NPC cell lines 5-8F and 6-10B were derived from the same NPC cell line SUNE-1. Although sharing almost the same genetic background, the two NPC cell lines have different metastatic capability, for 5-8F cell line had high metastasis potential, while 6-10B cell line was non-metastatic [38].

Patients and tissues
Six chronic nasopharyngitis (CN) biopsies and 28 primary poorly-differentiated NPC biopsies were obtained from CN and NPC patients with consent before treatment at Hunan Tumor Hospital (Changsha, Hunan, China), Xiangya Hospital of Central South University (CSU), the Second Xiangya Hospital of CSU and the Third Xiangya Hospital of CSU (Changsha, Hunan, China) in 2006 and 2007.

A total of 120 paraffin-embedded specimens, including 7 normal nasopharyngeal epithelia samples, 24 mild or moderate atypical hyperplasia samples, 9 severe atypical hyperplasia samples and 80 NPC samples, were supplied by Hunan Tumor Hospital and the Second Xiangya Hospital of CSU. All the specimens were stained with haematoxylin and eosin (HE) for histological examination and reviewed by an otorhinolaryngologic pathologist. The present study was approved ethically by Cancer Research Institute review board of CSU. All patients provided informed written consent.

Immunohistochemistry (IHC) staining
Investigating the expression of BCAT1 in different stages of precancerous lesions can help us evaluate the significance of this gene in NPC pathogenesis. Therefore, we used IHC method to analyze the expression of BCAT1 protein in the normal nasopharyngeal epithelia including pseudo-stratified ciliated epithelium and stratified epithelia, low-to-moderate grade atypical hyperplasia tissues, high-grade atypical hyperplasia tissues and NPC tissues, according to the protocol described in our previously published paper [39]. Meanwhile, the co-expression of BCAT1 and c-Myc in NPC tissues was also detected by IHC. Incubation with anti-BCAT1 (BD, Franklin Lakes, NJ) or anti-c-Myc (Calbiochem, Darmstadt, Germany) was carried out overnight at 4°C. Semi-quantitative assessment of BCAT1 and c-Myc immunostaining was performed by consensus and comprised both intensity of staining (0, 1, 2, or 3) and extent of staining (0, 0%; 1, <10%; 2, 10-50%; 3, >50%). The scores for the intensity of staining and extent of staining were multiplied to give a weighted BCAT1 or c-Myc score for each case (maximum possible, 9). The cases with at least moderate staining intensity (2 or 3) in a minimum of 10% of tumor cells were regarded as BCAT1 or c-Myc positive (++, +++, total weighted score of >4 out of 9), while the cases with weighted score of 0 (−) or 1–3 (+) were regarded as BCAT1 or c-Myc negative. BCAT1 immunostaining in normal or hyperplastic nasopharyngeal epithelia was similarly assessed. All of the biopsy samples were detected under the exactly same condition.
Detection of exon mutation of BCAT1 in NPC tissues

The primers for all the 11 exons of BCAT1 were designed by Primer 5 software and synthesized by Invitrogen (Shanghai, China). PCR was carried out using the genomic DNA from NPC tissues and the matched blood samples as templates. Then the PCR products were sequenced after being purified. The primer sequences are listed in Table 3.

Real-time quantitative PCR (qPCR) and reverse transcription PCR (RT-PCR)

The qPCR was performed on a Bio-Rad iQ5 system (Hercules, CA) with SYBR Green I PCR kit (TaKaRa, Dalian, China) to quantitatively analyze BCAT1 amplification in NPC tissues. Three microsatellite loci located in the BCAT1 gene were selected for this analysis. Gene amplification was set as 2-ΔΔC >2.0 compared with control. Semi-quantitative RT-PCR was performed to detect the mRNA expression levels of BCAT1 and c-Myc in CN tissues, NPC tissues and cell lines. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from DNase I-digested RNA (2 μg) using oligo(dT) as the primer with a commercially available reverse transcription system (Promega, Madison, WI) according to the manufacturer’s protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. Due to small size of NPC biopsies, the NPC samples for RT-PCR and IHC were not the same batch. The primer sequences for qPCR or RT-PCR are listed in Table 4.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed with EZ-ChIP™ kit (Millipore, Darmstadt, Germany). Two specific primers (5'-TGGCA TAGCACTGAAAGG-3' and 5'-CTGACTGGCAGTGTGTTG-3') were used to amplify a 193 bp fragment containing the predicted c-Myc binding site in the BCAT1 regulatory region. As a negative control, GAPDH was also amplified with the corresponding primers (5'-CGACC ACTTGTGTCACATCCA-3' and 5'-AGGGGTCTACATGCACA-3').

Plasmids and recombinants

The plasmids including pGL3-control, pGL3-promoter and pRL-TK used for luciferase reporter gene expression analysis were purchased from Promega Ltd. Vector for knocking down c-Myc expression (pRNA-T6.1/Si-c-Myc) and c-Myc expression vector (pCMV-HA/c-Myc) were both presented by Dr. Huaying Liu from our institute. For cloning pRNA-T6.1/Si-c-Myc, a vector expressing shRNA, an oligonucleotide encoding a stem-loop structure targeting c-Myc with the targeting sequence AGACTCTGACACTGTCCA, was designed and then subcloned into the pRNA-T6.1 vector (Genscript, Piscataway, NJ) under the control of the U6 promoter.

Table 3 Primers for amplifying 11 exons of BCAT1

| Primer | Sequence (5'-3') | Product size (bp) |
|--------|------------------|------------------|
| Exon 1 | F-GGGGAGCAGCAGGCTTAGT  R-GAGTGGAGGTTAAACCGAAA | 456 |
| Exon 2 | F-TACCCACCTGCATTTACTT  R-TCAACGTGCTTTGTTTCTC | 583 |
| Exon 3 | F-TAATCTAGGCCAGGCAAAGT  R-GTACCCACAGTGAAGTGC | 311 |
| Exon 4 | F-GTACCCACAGTGTAAGGGA  R-GTATGGTAAGAGGTAGGGA | 251 |
| Exon 5 | F-AAGTATGGTAATAGCTCCTG  R-ATGGCACTAACTAAATGGTC | 352 |
| Exon 6 | F-AGATGTTAGATAGGGAGAAG  R-GTATGGTAAGGAGTGAAGGA | 417 |
| Exon 7 | F-GGATGTTAGATAGGGAGAAG  R-GTATGGTAAGGAGTGAAGGA | 250 |
| Exon 8 | F-ATGCTATGATTGAGAAGG  R-AGACATCGTGGGAAGTAATT | 478 |
| Exon 9 | F-GCCACTTCCAGGTCTTCCC  R-GCATCTGTTGGCTGGGTC | 385 |
| Exon 10 | F-CTTCAGTGGAATTGCCTTAG  R-TTCCCCATTTGCTCTGTTG | 375 |
| Exon 11 | F-TCAAGGCAAGGGGGAGCAC  R-GTAGCCAAAGAAATCTATCACA | 251 |

Table 4 Summary for primer sequences and product sizes

| Primer | Sequence (5'-3') | Product size (bp) |
|--------|------------------|------------------|
| Primers for RT-PCR | | |
| GAPDH | F-5'-CCACCCCATGGCAAATTCCATGGCA-3'  R-5'-TCTAGACGGCAGGTCAGGTCCACC-3' | 550 |
| BCAT1 | F-5'-CCAAGAACCTGTGCTTTGTA-3'  R-5'-TGGAGGAGTTGGCAGTCTCACC-3' | 305 |
| c-Myc | F-5'-CTCACCCCTTCTCAACGACAGC-3'  R-5'-TTCTTCTAGACAGTGCTGTC-3' | 179 |
| KRAS | F-5'-GCAGACAGCAAGACAGGGTG-3'  R-5'-TGGAGGAGTTGGCAGTCTCACC-3' | 264 |
| MCAM | F-5'-ACCACTTGACACTCCAGT-3'  R-5'-TGCTTCTCCAGACAGGATG-3' | 213 |
| Primers for microsatellite loci of BCAT1 | | |
| D12S1435 | F-CTTGTGCAACCCTCCCAC  R-ATATGTGCTGTGAATACATC | 198 |
| D12S1617 | F-AGCCTGAGGGGCCAC  R-TGGACCACTTTGATAAGAAACA | 259 |
| RH44650 | F-AAGAATGTGTCTAAGGGCTA  R-CTCATGCTCTGAAGGTTTG | 146 |
were plated in 24-well plates at a density of 1 × 10⁴ cells/
c8F-Si-c-Myc cells with inhibited DNA sequencing.
constructed. Key regions in all constructs were verified by DNA sequencing.

**Knockdown of c-Myc in NPC cell lines**
5-8F-Si-c-Myc and 6-10B-Si-c-Myc cell lines with suppressed endogenous c-Myc expression were established by introducing pRNAT-U6.1/Si-c-Myc vector into 5-8F cells and 6-10B cells, respectively. For comparison, 5-8F-vector and 6-10B-vector cell lines were also yielded by transfecting pRNAT-U6.1 blank vector into 5-8F cells and 6-10B cells. Stable transfection was performed using Lipofectamine™ 2000 reagent (Invitrogen) following the manufacturer’s instructions. G418 (500 μg/ml, Millipore) was used to select the stable clones. The mRNA expression levels of c-Myc, BCAT1, KRAS and MCAM in NPC cell lines were detected by RT-PCR as previously described. Since the optimal PCR amplification parameters of them were not identical, we examined their expressions in different tubes. GAPDH was used as endogenous reference gene for normalizing variance in the quality of RNA and the amount of input cDNA. The same volume of PCR products was used to be analyzed by electrophoresis on the same agarose gel. The intensity of each band was measured by Image Master VDS (Pharmacia Biotech, Piscataway, NJ) and was analyzed by Bandleader software version 3.0. The expression levels of c-Myc, BCAT1, KRAS and MCAM in NPC cells were evaluated after they were normalized by transforming them into the ratio of the band intensity of each gene over that of GAPDH of the same samples. Each sample was repeated in triplicate. The primer sequences for RT-PCR are listed in Table 4.

**Luciferase activity assay**
COS7 cells with no endogenous c-Myc expression, 5-8F-Si-c-Myc cells with inhibited c-Myc expression, 5-8F-vector cells with endogenous c-Myc expression, were plated in 24-well plates at a density of 1 × 10⁵ cells/well. After 24 hrs, pGL3-233/-41 (or pGL3-233/-41-M), pCMV-HA/c-Myc and pRL-TK vectors were introduced into COS7 cells at a ratio of 10:10:1, and pGL3-233/-41 (or pGL3-233/-41-M) and pRL-TK vectors were co-transfected into 5-8F-Si-c-Myc and 5-8F-vector cells at a ratio of 10:1 by Fugene 6 transfection reagent (Roche, Switzerland). Another 36 hrs later, cells were washed twice, suspended in 500 µl reporter lysis buffer (Promega), and then the firefly luciferase activity was measured using the dual luciferase reporter assay system and a GloMax 20/20 luminometer (Promega) according to the manufacturer’s protocol. The Renilla luciferase vector pRL-TK (Promega) was co-transfected to standardize transfection efficiency in each experiment. As a positive control, the pGL3-control vector was also co-transfected into COS7 cells with pCMV-HA/c-Myc and pRL-TK vectors at a ratio of 10:10:1 or co-transfected into 5-8-Si-c-Myc and 5-8F-vector cells with pRL-TK vector at a ratio of 10:1.

**Colony formation assay**
Colony formation assay was conducted as described in our published paper [12] with minor modification. 5-8F, 5-8F-shBCAT1 and 5-8F-vector cells were seeded in six-well plates at the density of 700 cells per well. 5-8F-shBCAT1 and 5-8F-vector cells were established in our previous work [16]. After incubation for 8 days at 37°C in a 5% CO₂ incubator, the cells were fixed with methanol and stained with crystal violet. Colonies containing at least 50 cells were counted under inverse microscope (Nikon, Japan). Colony formation ratio was also calculated.

**Cell migration and invasion assays**
The *in vitro* migration and invasion abilities were compared between 5-8F-shBCAT1 and 5-8F-vector cells by using transwell chambers and matrigel-coated invasion chambers (Corning, Tewksbury, MA). For invasion assay, 8 μm pore transwell inserts coated with matrigel in cold serum-free media were seeded with 5 × 10⁴ cells per well and incubated for 48 hrs. Non-invasive cells on the upper surface of the filter were removed by wiping with a cotton swab, and cells that migrated through the membrane and stuck to the lower surface of the membrane were fixed with 10% paraformaldehyde and stained with 0.1% hexamethylenepararosaniline for 30 mins. For quantification, the cells were counted in five predetermined fields under a microscope. Data were expressed as the average number of cells migrating through the filters. The procedures of migration assay were similar to those described in matrigel invasion assay except there was no matrigel, the incubation time was 18 hrs and the fixative was methanol.

**Bioinformatic analysis**
Some bioinformatics tools such as Neural Network Promoter Prediction (NNPP) (http://www.fruitfly.org/seq_tools/promoter.html) and Transcription Element Search Software (TESS) (http://www.cbil.upenn.edu/tess) were used to predict the possible regulatory relationship and interaction mode between c-Myc and BCAT1. SNPs database (http://www.ncbi.nlm.nih.gov/snp/) was utilized to discriminate between mutation and SNP of BCAT1.
Statistical analysis
Statistical analysis was performed using Wilcoxon rank sum test, chi-square test, and student t-test. In all analyses, SPSS 13.0 statistical software was used and the statistical significance level was set at P < 0.05.

Abbreviations
CGH: Comparative genomic hybridization; ChIP: Chromatin immunoprecipitation; CN: Chronic nasopharyngitis; CSU: Central South University; HE: Haematoxylin and eosin; IHC: Immunohistochemistry; NNPP: Neural network promoter prediction; NPC: Nasopharyngeal carcinoma; qPCR: Real-time quantitative PCR; SNP: Single nucleotide polymorphism; TESS: transcription element search software; TSG: Tumor suppressor gene; TSS: Transcription start site.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
RC designed the general study, wrote the protocols, revised the manuscript and provided the funding. ZW and FX performed most of the experiments. JX, HW, Liu Z, Li Z, ZL and WL contributed to administrative, technical or material support (such as clinical samples collection). ZB, SJ, LJ and LY were in charge of the literature searches, analyses and partial experiments. WL undertook the statistical analysis. ZW and HW drafted the manuscript. YK provided critiques of the manuscript. All authors read and approved the final manuscript.

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