available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

ENO1, a potential prognostic head and neck cancer marker, promotes transformation partly via chemokine *CCL20* induction

Sen-Tien Tsai [a,b], *I-Hsiu Chien* [d,h], *Wen-Hao Shen* [d,h], *Yi-Zih Kuo* [e], *Ying-Tai Jin* [c,d], *Tung-Yiu Wong* [d], *Jenn-Ren Hsiao* [a], *Hsing-Ping Wang* [f], *Neng-Yao Shih* [g], *Li-Wha Wu* [f,*]

[a] *Department of Otolaryngology, National Cheng Kung University Hospital, Tainan 70428, Taiwan, ROC*
[b] *Tainan Hospital, Department of Health, Tainan 70043, Taiwan, ROC*
[c] *Department of Pathology, National Cheng Kung University Hospital, Tainan 70428, Taiwan, ROC*
[d] *Institute of Oral Medicine, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan, ROC*
[e] *Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan, ROC*
[f] *Institute of Molecular Medicine, College of Medicine, National Cheng Kung University, 1 University Road, Tainan 70101, Taiwan, ROC*
[g] *National Institute of Cancer Research, National Health Research Institute, Tainan 70456, Taiwan, ROC*

ARTICLE INFO

Article history:
Received 17 November 2009
Received in revised form 10 March 2010
Accepted 18 March 2010

Keywords:
Glycolysis
Enolase
ENO1
Head and neck cancer
CCL20

ABSTRACT

The success of using glycolytic inhibitors for cancer treatment depends on studying the individual role of frequently deregulated glycolytic genes in cancer. This report aims to study the prognostic implication, and determine the cellular role and action mechanism of glycolytic *ENO1* overexpression in head and neck cancer. The relationship of *ENO1* mRNA expression in 44-pair clinical specimens with patient clinicopathologic characteristics was analysed by semi-quantitative RT-PCR, Kaplan–Meier survival curve and Cox model analyses. Following ectopic *ENO1* expression or knockdown, we studied the proliferative, migratory, invasive, colony-forming and tumourigenic abilities of *ENO1*-genetically altered cells. DNA microarray analysis was used to identify downstream targets responsible for the *ENO1* action in the cells. The expression of *ENO1* mRNA was increased in 68% of tumour (T) specimens when compared to their normal (N) counterparts, and positively associated with clinical progression ($p < 0.05$). High *ENO1* expression ($T/N \geqslant 2$) was frequently observed in the patients with large primary tumours, late clinical stages or advanced neck metastasis. Moreover, high *ENO1* patients had significantly poorer clinical outcomes than low expressers ($T/N < 2$). Ectopic *ENO1* expression stimulated cell transformation, invasion and tongue tumour formation. *ENO1* knockdown abrogated the stimulation. Suppression of *ENO1*-induced proinflammatory *CCL20* chemokine expression significantly attenuated its stimulatory effects on cell transformation and invasion. A concordant expression of *ENO1* and *CCL20* was validated both in *ENO1*-expresing cells and in clinical specimens. Together, we demonstrate a prognostic role of *ENO1* overexpression in head and neck cancer and *ENO1*-mediated promotion of cell transformation and invasion partly via induced *CCL20* expression.

* *Corresponding author:* Tel.: +886 6 2353535x3618; fax: +886 6 2095845.
E-mail address: liwhawu@mail.ncku.edu.tw (L.-W. Wu).
[h] These authors contributed equally to this work.
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2010.03.018

1. Introduction

During tumour formation and expansion, tumour cells must increase glucose metabolism to support the increased demand of energy necessary for unrestricted growth.[1] In addition to accelerated glucose metabolism, aberrant microvasculature associated with tumour is ineffective at delivering oxygen to tumour cells, leading to hypoxia. Hypoxia is another common feature of solid tumours.[2] Under this condition, activation of hypoxia-inducible factor (HIF), particularly HIF1, drives the expression of a myriad of genes controlling multiple cell functions including those involved in glycolysis.[3] Consistent with the hypoxic nature of tumours, overexpression of glycolytic genes has been found in 24 human cancer types, including head and neck cancer.[4]

Recent studies indicate that some glycolytic enzymes are complicated, multifaceted proteins rather than simple components of the glycolytic pathway.[5] Enolase, catalysing the conversion of 2-phosphoglycerate into phosphoenolpyruvate, is one glycolytic enzyme found in multiple, distinct subcellular compartments.[6] Cell surface enolase even acts as a receptor for plasminogen and the binding activates plasminogen,[7] a key molecule in the protease system involved in tumour cell invasion and metastasis.[8] In line with this observation, the interaction of cell surface-associated enolase with plasminogen promotes monocyte migration and invasion to an acutely inflamed lung tissue.[9]

Enolase is encoded by three independent loci, *ENO1*, *ENO2* and *ENO3*, in mammals. The product of *ENO1* gene is present in almost all adult tissues, whereas those from *ENO2* and *ENO3* are, respectively, found in neuron/neuroendocrine tissues and muscles.[10] Enolase 1 is the frequently deregulated isoform in many cancer types.[11] At least three mechanisms potentially account for the alteration. First, *ENO1* is located in the chromosomal region 1p36, a frequently rearranged or deleted region in human malignancy. A novel and frequent amplification unit centring in the vicinity of *ENO1* gene was found in lung cancer.[12] Second, HIF1 induces *ENO1* transcription via hypoxia response elements in the promoter.[13] Third, the expression of *ENO1* mRNA was elevated in *c-MYC*-overexpressing murine cells, suggesting a positive role of oncogenic *c-MYC* in the increase of *ENO1* expression.[14]

Contrasting roles for *ENO1* in tumourigenesis have been reported. Downregualtion of enolase 1 protein in non-small lung cancer cells indicates a tumour suppressor function of *ENO1*.[15] In line with this notion, introduction of *in vitro* transcribed *ENO1* mRNA into neuroblastoma or embryonic kidney 293 cells induced cell death.[16] By contrast, autoantibodies to enolase 1 were detected in sera and pleural effusion of non-small cell lung cancer patients, and high level of enolase 1 expression was associated with poor prognosis in lung cancer.[17,18] Enolase 1 is highly expressed in metastatic head and neck cancer cells compared to their non-metastatic counterparts, suggesting an oncogenic role of enolase.[19] Further studies are thus needed to clarify the exact functions of enolase 1 in different cancer types.

Head and neck cancer is the sixth most frequent cancer worldwide and the five-year survival rate is amongst the lowest of the major cancers.[20] More than 90% of head and neck cancer occurs in the oral cavity.[21] The presence of *ENO1* transcripts in oral cancer and a preferential location of its gene product in the basal cell layer of stratified squamous epithelium suggest a role of *ENO1* in oral carcinogenesis.[22] Oral cancer was used as a model to study the causal relationship between *ENO1* deregulation and head and neck cancer, and the identity of *ENO1* downstream targets that may be involved in this action. We genetically manipulated *ENO1* expression in oral cancer cells and confirmed the role of *ENO1* expression both *in vitro* and *in vivo*. The clinical significance of *ENO1* deregulation and the underlying mechanism responsible for the *ENO1* action in head and neck cancer cells were also presented.

2. Patients and methods

2.1. Cell culture

Normal oral keratinocytes (NOKs) from gingival tissues of healthy individuals with informed consent were grown in calcium-free Epilife Medium supplemented with human keratinocyte growth supplement (Cascade Biologics, Portland, OR, USA). Dysplastic oral keratinocyte (DOK) was maintained in DMEM with 10% FBS and 5 μg/ml hydrocortisone. Five oral cancer cell lines, CAL-27, OC-2, OC-3, OEC-M1 and HSC-3, were maintained as described.[23]

2.2. Patient specimens

Frozen tissues of surgically resected tumours and adjacent normal tissues from 2001 to 2007 were obtained with informed consent from the archives of Tissue Bank at the National Cheng Kung University Hospital. Their use for this study was approved by the Institutional Review Board. The study population included 41 males and 3 females with a median age of 50 years and clinicopathological characteristics in Table S1. Each characteristic between high and low *ENO1* groups was compared by Chi-square test. Kaplan–Meier survival method and log-rank test were used to evaluate the relation of *ENO1* expression and patient survival. Cox regression model was used to assess the independent value of potential prognostic factors.

2.3. Online supplementary methods

All the other methods are in the supplementary materials and methods.

3. Results

3.1. Increased ENO1 expression correlates with poor prognosis of oral cancer patients

To study whether *ENO1* was overexpressed while avoiding the cross-reactivity of anti-enolase 1 antibodies in human clinical specimens, we analysed the level of *ENO1* mRNA in 44 surgical pairs of tumour (T) and adjacent normal (N) tissues by

semi-quantitative RT-PCR. The expression of *ENO1* mRNA was increased in 68% of the tumour specimens compared to that of their normal parts (T/N > 1 in 30 patients). Moreover, *ENO1* mRNA expression level significantly increased with oral cancer progression (Fig. 1A, $p < 0.05$).

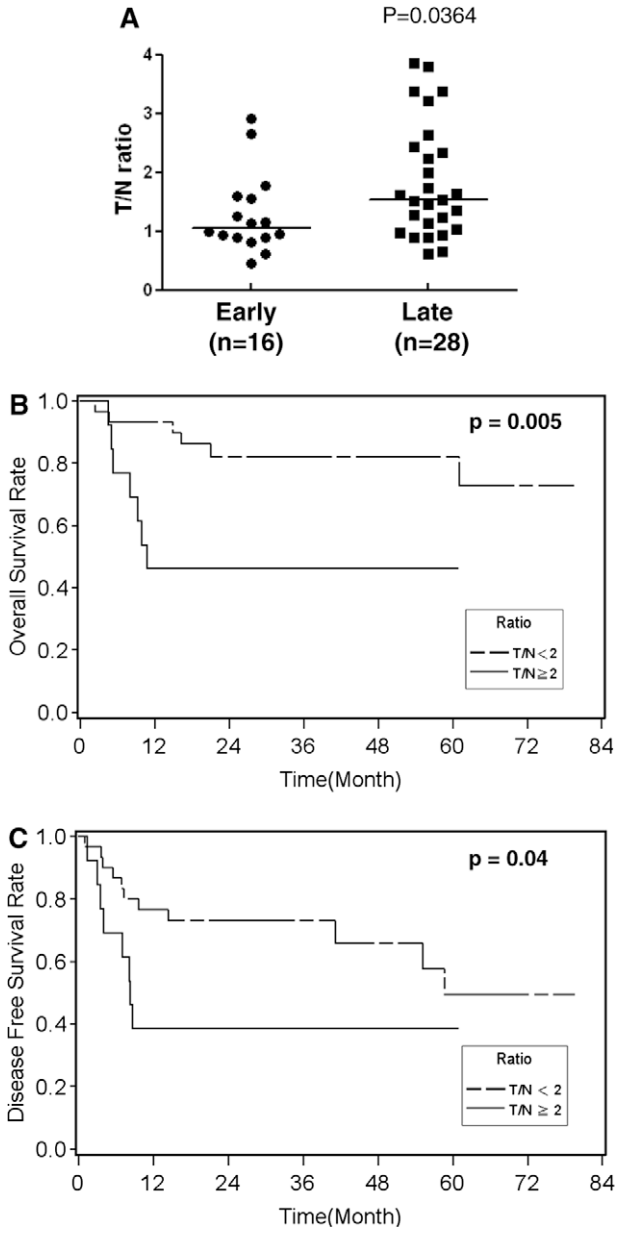

Fig. 1 – *ENO1* mRNA expression increased with clinical progression of oral cancer and its high expression was associated with poor clinical outcomes. (A) *ENO1* mRNA levels in 44-pair surgical specimens including tumour (T) and normal (N) tissues were measured by RT-PCR. Following *GAPDH* normalisation, *ENO1* mRNA expression ratio in each patient was expressed as T/N and a median ratio for each stage was indicated. Early, clinical stages I and II. Late, clinical stages III and IV. (B) OS and (C) DFS in these patients were stratified by *ENO1* mRNA expression status, T/N ⩾ 2 and T/N < 2.

We divided these patient specimens into high (T/N ⩾ 2) and low groups (T/N < 2) based on *ENO1* expression ratio. No statistical difference was observed in age, tumour site and size, differentiation, neck metastasis and recurrence between two groups (Table S1). However, high *ENO1* expression was frequently observed in patients with large primary tumours ($p = 0.07$), late clinical stages ($p = 0.09$) or positive neck metastasis ($p = 0.06$). Advanced neck metastasis predominantly occurred in high *ENO1* group (Cochran–Armitage trend test, $p = 0.03$, Table S2). Kaplan–Meier survival analysis revealed that oral cancer patients with high *ENO1* expression had both poor overall and disease-free survival (Fig. 1B and C, $p < 0.05$). We used univariate and multivariate Cox proportional-hazard models to estimate relative risk of developing the diseases. High *ENO1* patients are more likely to die from the disease (Hazard ratio: 4.5, 95% confidence interval, $p = 0.01$) and develop the recurrence (Hazard ratio: 2.5, 95% confidence interval, $p = 0.05$) in univariate but not in multivariate analyses. Together, *ENO1* mRNA expression status is a potential prognostic marker for the clinical outcomes of oral cancer patients.

3.2. *Frequent ENO1 overexpression in oral cancer lines*

The level of enolase in normal NOK cells, precancerous DOK and oral cancer lines, OEC-M1, OC-3, CAL-27 and HSC-3 was examined by Western blot analysis using anti-enolase antibodies (Fig. 2A). The level of enolase was the lowest in NOK, slightly increased in precancerous DOK cells but markedly increased in oral cancer lines, suggesting a positive role of enolase in cancer. To study enolase isoform distribution in these cell lines, antibodies specific to enolase 1, 2 or 3 were used for Western blot analysis. Amongst the isoforms, enolase 1 was not only differentially expressed but also expressed at higher level in oral cancer cells than in NOK (Fig. 2B). Enolase 2 was

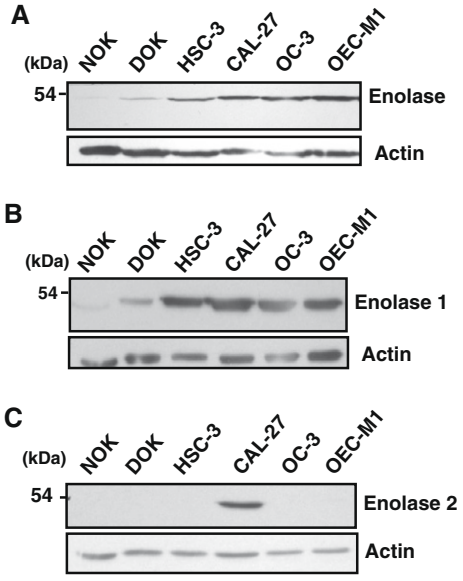

Fig. 2 – Enolase 1 overexpression in oral cancer lines. Western blot analysis of protein lysates from subconfluent NOK, DOK and 5 other oral cancer lines using (A) C19 anti-enolase, (B) anti-enolase 1, or (C) anti-enolase 2 antibodies. β-actin, a loading control.

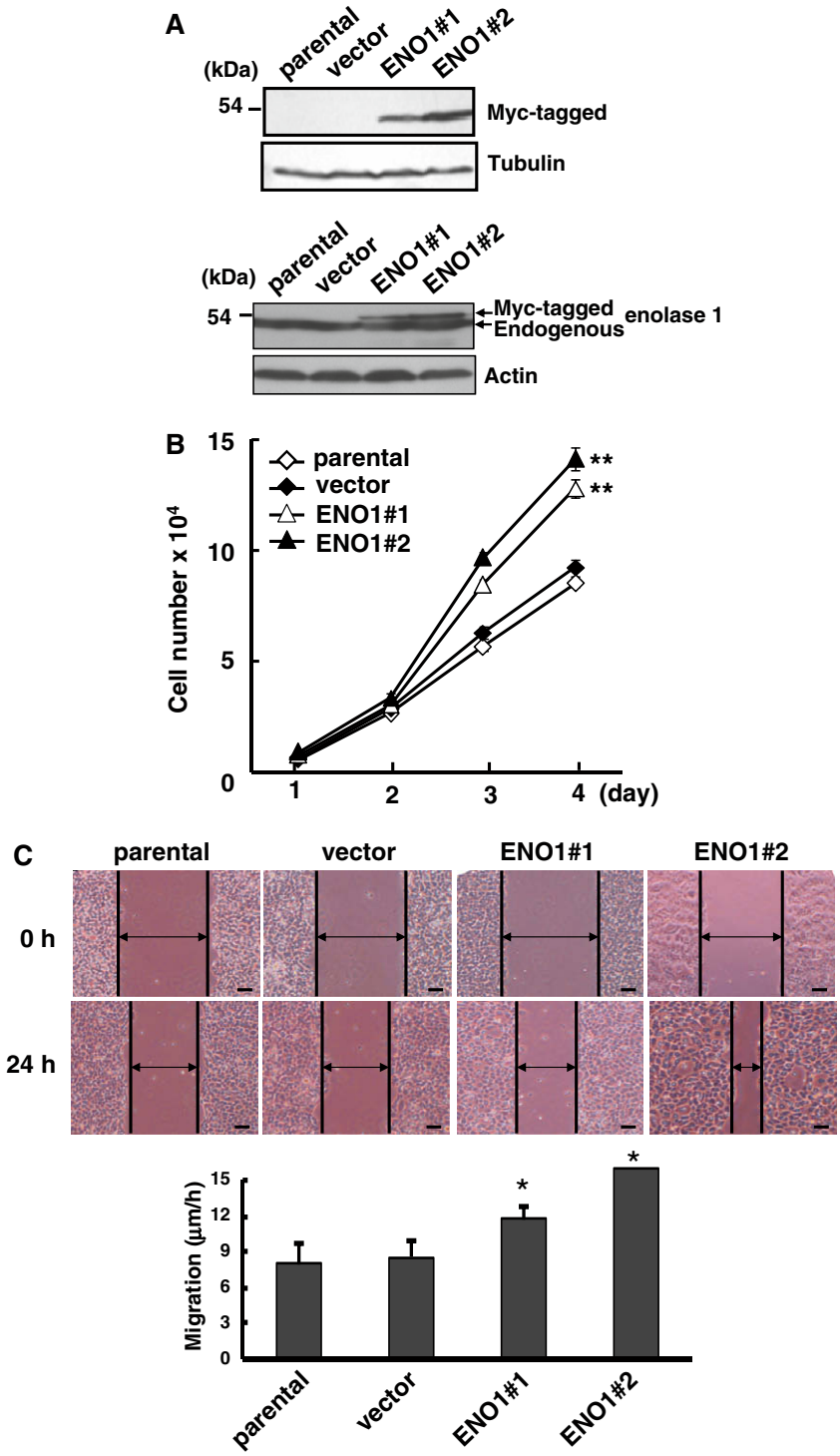

Fig. 3 – Ectopic *ENO1* expression increased cell transformation and invasion. (A) Western blot analysis of protein lysates from parental, vector, ENO1#1 and ENO1#2 cells using anti-Myc (top), anti-enolase 1 (bottom). Tubulin or actin, loading controls. (B) Parental, vector, ENO1#1 and ENO1#2 cells are enumerated every 24 h for 4 days. (C) Representative scraping wounds formed by parental, vector and ectopic *ENO1*-expressing ENO1#1 and ENO1#2 cells were measured before wounding (0 h) and 24-h post-wounding (24 h) (top). Migration rate was expressed as μm/h (bottom). *$p < 0.05$ versus vector. Scale bar, 100 μm. (D) Ten HPF (400×) of invasive blue cells of parental, vector, ENO1#1 or ENO1#2 clones were enumerated. (E) Total purple colonies formed by parental, vector, ENO1#1 or ENO1#2 cells were individually quantified. *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ versus vector.

only expressed in CAL27 cells (Fig. 2C). Enolase 3 was not detected in any of tested cell lines (data not shown). Together, enolase 1 but not the other two isoforms was frequently over-expressed in oral cancer cells.

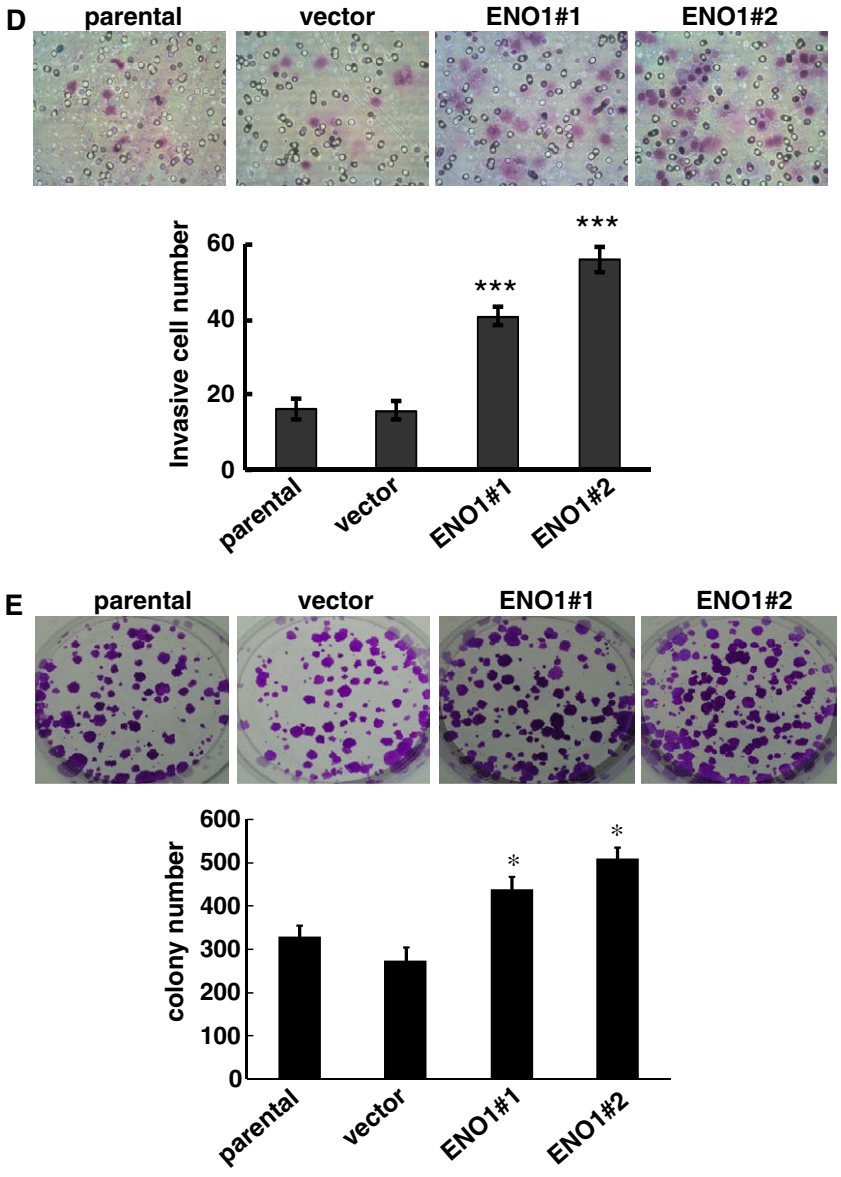

Fig. 3 *(continued)*

3.3. *Ectopic ENO1 expression in oral cancer lines*

To study the effect of increased *ENO1* expression in oral cancer cells, CAL27 cells with a better cloning efficiency were used as a host for establishing ectopic *ENO1*-expressing stable clones. Two *ENO1*-stable expressing clones together with parental and vector control were analysed for the expression of ectopic Myc-tagged and endogenous enolase 1. In addition to similar levels of endogenous enolase 1, ENO1#2 clone expressed a higher level of Myc-tagged enolase 1 than ENO1#1 clone (Fig. 3A). No obvious morphological change was observed between vector and ectopic *ENO1*-expressing cells (data not shown). ENO1#1 and ENO1#2 together with parental and vector control clones were used for studying the effect of increased *ENO1* expression in cellular and tumourigenic behaviours.

3.4. *Ectopic ENO1 expression increased cell transformation*

To examine the impacts of ectopic *ENO1* expression on the transforming behaviours of transfected cells, parental, vector control and ectopic *ENO1*-expressing clones (ENO1#1 and ENO1#2) were used for growth rate, wound healing, Trans-well invasion and colony formation assays. There was no obvious difference in the cellular behaviours of parental and vector control cells (Fig. 3B–E). However, ectopic *ENO1* expression significantly promoted the ability of cancer cells to proliferate (Fig. 3B), migrate (Fig. 3C), invade Matrigel (Fig. 3D) and form colonies (Fig. 3E), when compared to the vector control cells. ENO1#2 cells with higher *ENO1* expression had a greater transforming ability than ENO1#1 cells. Together, ectopic *ENO1* expression dose-dependently increased cell proliferation,

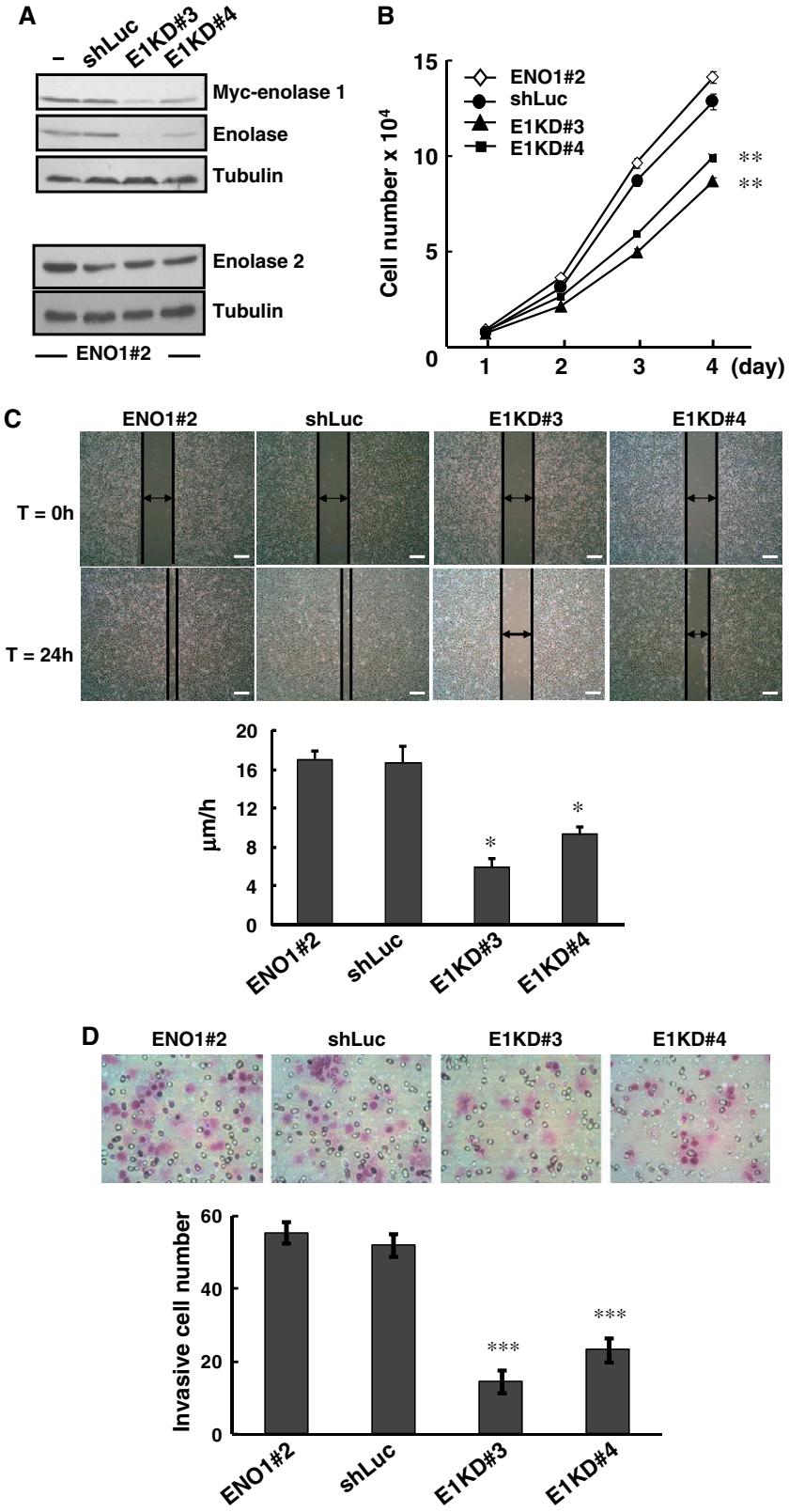

Fig. 4 – Specific *ENO1* knockdown reduced *ENO1*-mediated promotion on cell transformation. (A) Western blot analysis of Myc-tagged enolase 1 (top) and enolase 2 (bottom) expression in shLuc, E1KD#3 and E1KD#4 knockdown cells using anti-Myc, anti-enolase 1 or anti-enolase 2 antibodies. (B) Parental ENO1#2, control shLuc and knockdown cells, E1KD#3 and E1KD#4, seeded in growth medium were enumerated every 24 h for 4 days. (C) The migration rate of ENO1#2, shLuc and *ENO1* knockdown cells was measured by wound healing assay and expressed as μm/h. Scale bar, 150 μm. (D) Following migration assay, ten HPFs of invasive blue cells on the lower membrane side were enumerated. (E) Total colonies formed by parental ENO1#2, shLuc and *ENO1*-knockdown cells were individually quantified. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus shLuc.

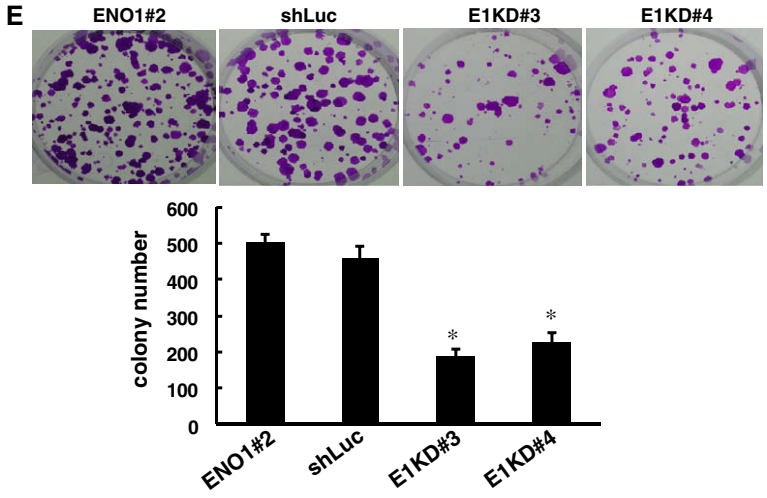

Fig. 4 *(continued)*

migration, invasion and colony-forming abilities, indicating a transforming effect of ENO1 overexpression.

3.5. *ENO1 knockdown reduced cell transformation*

The *in vitro* role of *ENO1* expression was further confirmed by knockdown approach. Following puromycin selection, two stable knockdown clones with 50–70% knockdown efficiency using high *ENO1*-expressing ENO1#2 cells as host cells were established and named as E1KD#3 and E1KD#4. ShRNA to luciferase (shLuc) served as an infection control. Western blot analysis confirmed the differential reduction of enolase 1 protein in both knockdown clones but not in parental ENO1#2 or shLuc cells (Fig. 4A). E1KD#3 has a lower enolase 1 expression than E1KD#4. The expression of enolase 2 protein was not affected in the *ENO1* knockdown cells, indicating the specificity of shRNA to *ENO1* expression.

Cell proliferation, wound healing, invasion and colony formation assays were used to examine the *ENO1*-knockdown effect on these cells. Control shLuc cells behaved similarly to the parental ENO1#2 cells (Fig. 4B–E). *ENO1* knockdown significantly attenuated the promoting ability of *ENO1* expression on cell proliferation (Fig. 4B), migration (Fig. 4C), invasion (Fig. 4D) and colony formation (Fig. 4E). The extent of reduction coincides with the intracellular level of enolase 1. The *ENO1*-knockdown effect was further validated in another oral cancer line OEC-M1 with endogenous enolase 1 expression. Greater than 50% of *ENO1*-knockdown significantly reduced OEC-M1 cells to proliferate, migrate, invade Matrigel and form colonies (Fig. S1). Together, an increase in *ENO1* expression indeed plays a promoting role for cell transformation.

3.6. *Ectopic ENO1 expression enhanced tongue tumour formation in nude mice*

To study if ectopic *ENO1* expression promoted tumourigenesis in oral cavity, a tongue tumour model was used to address the question. Five weeks following orthotopic injection of vector control, ENO1#1 or ENO1#2 cells into nude mouse tongues, we killed the mice and removed tongues from the oral cavity for weight measurement and HE staining. All tumour-bearing tongues weighed significantly heavier than normal tongue tissues ($p < 0.05$). The ectopic *ENO1*-expressing tumour-bearing tongues weighed heavier than vector control tumours, and ENO1#2 tumours with highest *ENO1* expression were significantly larger than vector control tumours (Fig. 5A). The tumours in the tongue were reminiscent of squamous cell carcinoma occurring in more than 90% of oral cancer.[21] Although invasions at both perineural regions and lymphatic vessels were detected in these tumours, lymphatic invasion was particularly enhanced by high *ENO1* expression (Fig. 5B and C). The relation of cell invasion and enolase 1 expression was further examined in NOK, DOK, OC-2, OC-3 and OEC-M1 using Western blot analysis and Trans-well invasion assay. As shown in Fig. 5D, increasing enolase 1 expression positively associated with cell invasiveness. Together, increased *ENO1* expression promoted not only tumour formation but also local invasion.

3.7. *Effect of increased ENO1 expression on global gene expression*

To understand the mechanism responsible for the transforming effect of increased *ENO1* expression, we used a HumanRef-8 v2 Expression BeadChip which contains 235,000 probes per array targeting genes and known alterative splice variants from RefSeq (Illumina, San Diego, CA) to compare the mRNA expression profile in ENO1#2 and vector control cells. Amongst 166 genes with 2 or greater fold change in ENO1#2 cells, 35 genes are associated with inflammation (data not shown). Amongst the 7 cytokines/chemokines induced by *ENO1* (Table S3), *CCL20*, frequently overexpressed in human cancer,[24,25] was the most expressed in these cells. Consistent with microarray data, more than 3-fold increase of *CCL20* mRNA was detected in *ENO1*-expressing cells by using semi-quantitative RT-PCR (Fig. 6A). Western blot analysis confirmed the release of CCL20 protein into the conditioned medium from five oral cell lines. The release of CCL20 protein was much higher in high enolase 1-expressing

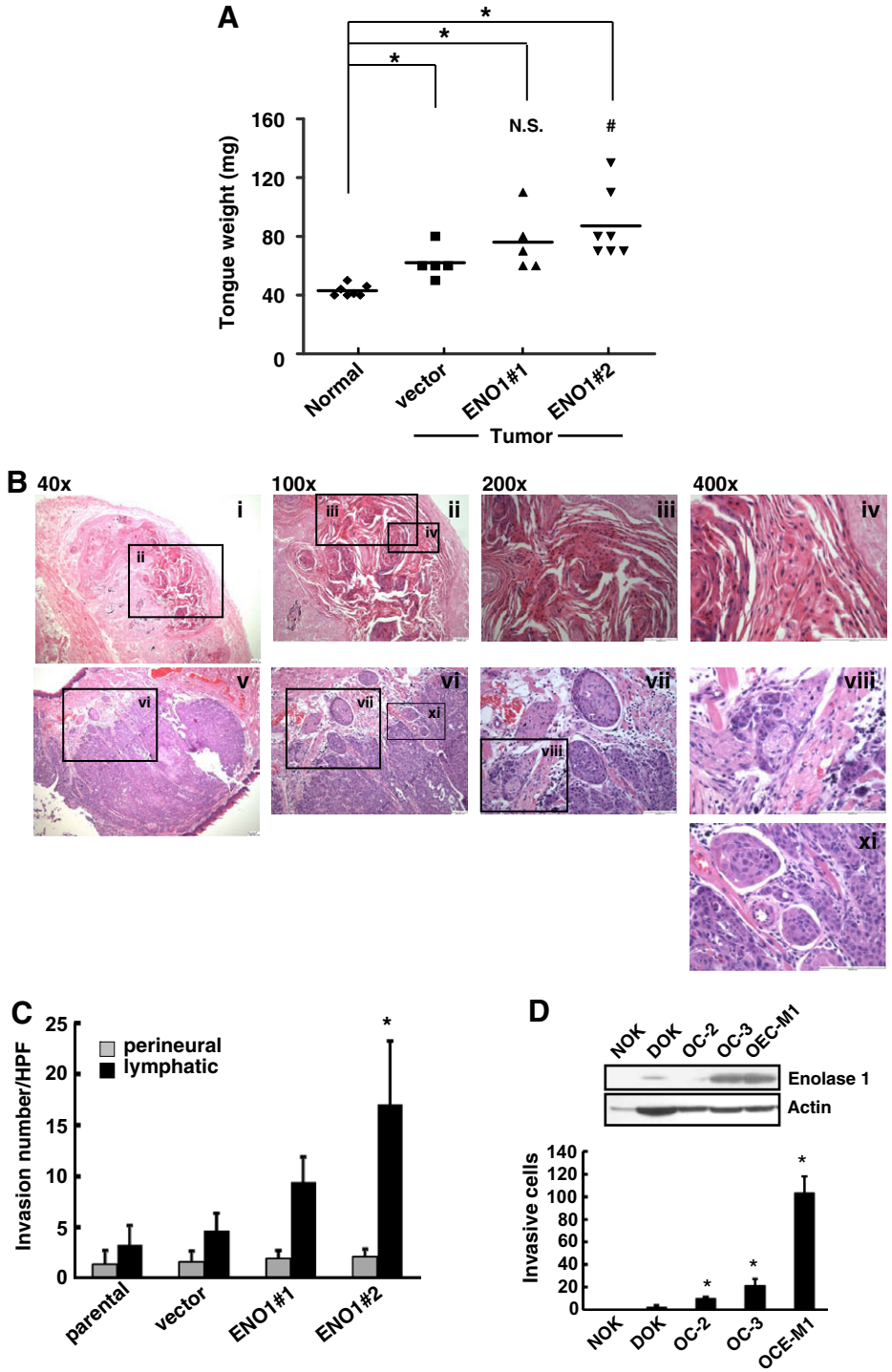

Fig. 5 – Ectopic *ENO1* expression promoted tongue tumour formation and invasion. (A) Tongues were removed from the oral cavity and weighed at 5 weeks post-injection. Normal tongue tissues at the same age serve as normal control. *$p < 0.05$ versus normal; N.S., not significant versus vector; #$p < 0.05$ versus vector. (B) Tissue sections were stained with H&E stain (i and v, 40×; ii and vi, 100×; iii and vii, 200×; iv, viii and xi, 400×). I–iv, vector control tumour; v–xi, ENO1#2 tumour. viii, perineural invasion; xi, lymphatic invasion. Insets are the images for higher magnifications. Scale bar, 100 μm. (C) Perineural and lymphatic invasion number in tumour sections, enumerated by microscopy (100×), was expressed as mean ± SEM. *$p < 0.05$ versus vector. Parental, CAL27 cells. (D) Enolase 1 protein expression in 5 cell lines was measured by Western blot analysis using anti-enolase 1 antibody (top) and the invasiveness of the same lines was examined by Trans-well invasion assay (bottom). Actin, a loading control. *$p < 0.05$ versus NOK.

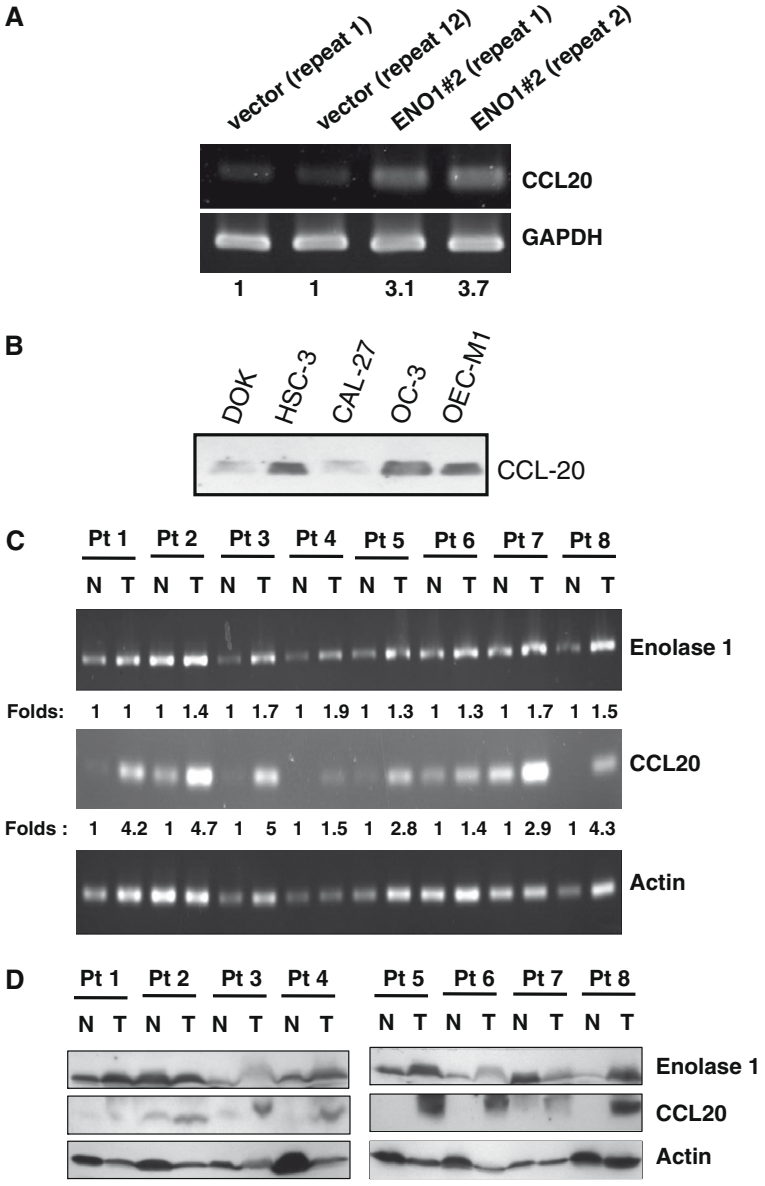

Fig. 6 – A concordant expression of *CCL20* and enolase 1 in enolase 1-expressing cells and clinical specimens. (A) The expression of *CCL20* and *actin* mRNA in vector control and ENO1#2 stable clones using RT-PCR analysis (repeats 1 and 2). Following normalisation with *GAPDH*, numbers on the bottom indicate fold changes of *CCL20* mRNA in ENO1#2 relative to vector control cells. (B) The expression of CCL20 protein in conditioned media prepared from the indicated cell lines was analysed by Western blot analysis using anti-CCL20 antibodies. (C) The mRNA expression of *ENO1*, *CCL20* and *actin* in 8 pair-wise (N versus T) patient specimens (Pt1-8) was measured by RT-PCR and expressed as fold change following the normalisation with actin. (D) Western blot analysis of enolase 1 and CCL20 protein in pairwise samples pt.1-8. Actin, a loading control.

OEC-M1 and OC3 lines than that in low enolase 1-expressing precancer DOK cells (Fig. 6B). This concordant relationship was further validated in 40 pair-wise surgical specimens by semi-quantitative RT-PCR. Spearman's rank correlation analysis showed a positive association of *CCL20* with *ENO1* mRNA expression in these tumour tissues (Spearman $\rho = 0.388$, $p = 0.013$, Fig. S2). Due to the limited number of frozen tissues collected from these patients, we confirmed this relationship in a separate cohort of 8 patients (Table S4) using RT-PCR and Western blot analysis . β-actin was used as a loading control to avoid the bias of using GAPDH as a control. We detected a corresponding increase of *CCL20* mRNA and protein in most of the oral cancer specimens with elevated enolase 1 expression when compared to adjacent normal tissues (Fig. 6C and D), suggesting that *CCL20* is a potential downstream target of *ENO1*.

3.8. *ENO1-mediated cell transformation partly via CCL20 up-regulation*

Although *CCL20* participates in inflammatory cell recruitment, it also promotes certain cancer cell proliferation and

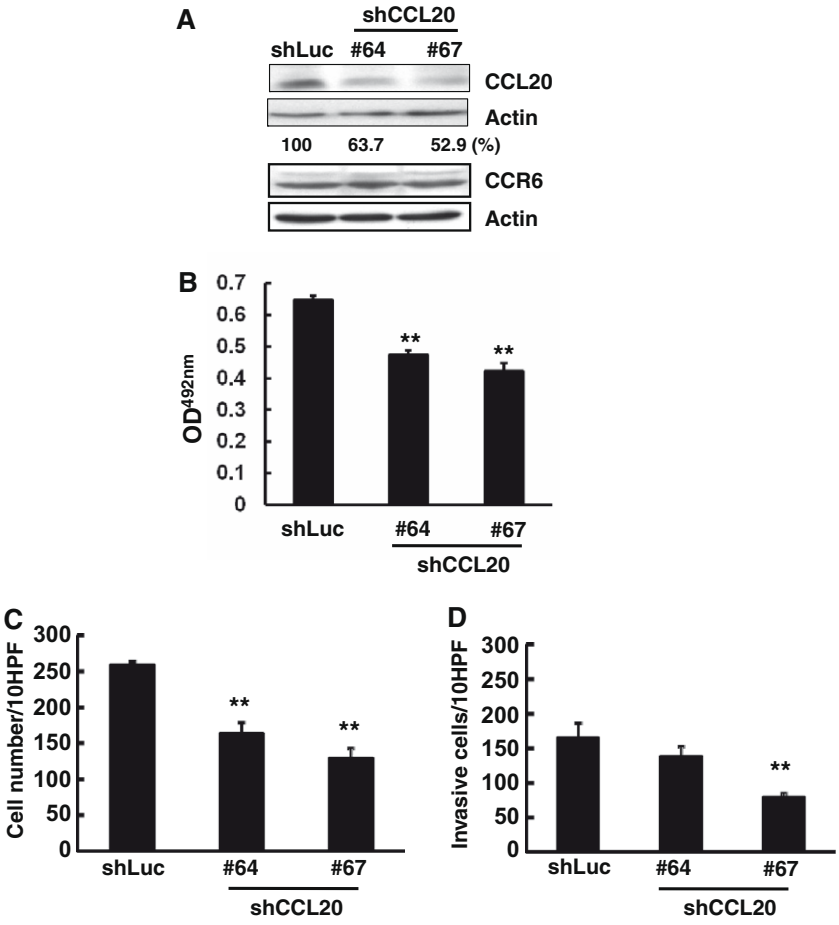

Fig. 7 – *ENO1*-mediated transformation via increased *CCL20* expression. (A) The expression of CCL20, CCR6 and actin protein in shLuc and CCL20-knockdown clones #64 and #67 using ENO1#2 as parental cells, detected by Western blot analysis. (B) Cell proliferation abilities of shLuc and *CCL20*-knockdown clones, measured by MTS kits, were expressed by absorbance at 492 nm wavelength. The migration rate (C) and invasiveness (D) of shLuc and CCL20-knockdown cells were, respectively, measured by Transwell migration and invasion assays. Ten HPFs (200×) were used to represent the number of migrated and invasive cells in each well. $^{**}p < 0.01$ versus shLuc cells.

invasion.[26–28] CCR6, the only receptor for CCL20, was abundantly expressed in oral cancer cells based on our microarray data (data not shown). To study the role of *CCL20* in *ENO1*-expressing cells, two shRNA clones, 64 and 67, with differential knockdown were used (Fig. S3). Etopic *ENO1*-expressing cells, ENO1#2, were used as host cells for knocking endogenous *CCL20* expression. The decrease of CCL20 protein but not its receptor, CCR6, was confirmed in these two clones using Western blot analysis (Fig. 7A). Cell proliferation, migration and invasion assays of *CCL20*-knockdown clones, #64 and #67, showed that suppressing *CCL20* expression dose-dependently reduced the promoting effect of *ENO1* overexpression on cell proliferation, migration and invasion (Fig. 7B–D, $p < 0.01$). Together, *CCL20*, a downstream target of *ENO1*, plays a positive role in the *ENO1*-mediated cell transformation.

4. Discussion

Enolase 1 was frequently overexpressed in head and neck cancer cells. The increase of *ENO1* mRNA expression associated not only with cancer progression but also with poor clinical outcomes in these patients. Moreover, neck metastasis severity significantly increased in the high *ENO1* group. Ectopic *ENO1* expression *in vitro* promoted cell proliferation, migration, invasion and colony formation. Specific *ENO1* knockdown abrogated *ENO1*-mediated stimulation of cell transformation. Moreover, the increase significantly promoted tumour growth and lymphatic invasion in tongue. *ENO1*-promoted cell transformation was partly via altering the expression of proinflammatory cytokine *CCL20*. Knocking *CCL20* down significantly compromised the stimulatory effect of *ENO1* overexpression on cell transformation. A positive association of *CCL20* with *ENO1* expression was observed in *ENO1*-expressing cells and validated in clinical specimens.

Although enolase is conventionally known as a glycolytic enzyme in the cytosol, the presence of enolase in distinct subcellular localisations other than cytosol indicates additional intracellular functions of enolase.[10] This notion was recently corroborated that cell surface enolase, a receptor for plasminogen, uses the plasminogen-binding sites to potentiate the directed migration and invasion of monocytic U937 cells.[7,9]

Consistent with diverse subcellular localisations of enolase in the cells, altering *ENO1* gene expression had profound impacts on cell proliferation, migration, invasion and transformation. Although *ENO1* overexpression induced apoptotic neuroblastoma or 293 cells,[16] no apoptotic effect of ectopic *ENO1* expression was detected in transiently or stably transfected oral cancer cells (data not shown). Moreover, the transforming ability was proportional to the expression level of *ENO1* in the cells. Although the exact function of enolase in different subcellular compartments of cancer cells remains elusive, our study of showing the transforming function of *ENO1* overexpression further supports that a tight regulation of enolase 1 is crucial for normal cell functions.

The production of phosphoenolpyruvate, the second of two high energy intermediates in ATP production, was catalysed by enolase during glycolysis.[29] Intracellular ATP levels in both *ENO1*-expressing and knockdown cells were measured by the luciferin-luciferase method. Although there was a trend of reduced ATP production in *ENO1*-knockdown cells depending on the knockdown efficiency, no significant change of ATP production was observed in ectopic *ENO1*-expressing cells when compared with vector control (data not shown). Therefore, the observed alterations induced by ectopic *ENO1* expression in the cellular behaviours were unlikely due to the changes of ATP production in these cells.

Metastasis is the main reason for the mortality of head and neck cancer. Understanding the molecular mechanism underlying metastasis holds a key for the development of anti-cancer treatment. Proteomic analysis identified up-regulation of enolase 1 in metastatic head and neck cancer, suggesting a role of enolase 1 in cancer invasion and metastasis.[19] Consistent with the notion, high *ENO1* expression was tightly associated with poor clinical outcomes in head and neck cancer. Moreover, high *ENO1* patients had a higher percentage of node positivity than low patients ($p = 0.06$, Table S1) and the severity of neck metastasis significantly increased in high group ($p = 0.03$, Table S2). Ectopic *ENO1* expression promoted tumour cell invasion through Matrigel *in vitro* and regional lymphatic invasion *in vivo*. Furthermore, the invasiveness of oral cancer cells positively correlated with the increased *ENO1* expression. In line with our observations, increased enolase 1 expression was previously shown to positively correlate with not only tumour size but also venous invasion in hepatitis C virus-related hepatocellular carcinoma.[30] Moreover, an increase of enolase 1 expression on the surface of monoctyes promotes their migratory and lung-infiltrating ability.[9] More studies are warranted to address if the same mechanism is also responsible for the invasiveness of *ENO1*-expressing cancer cells. Together, our *in vitro* and *in vivo* studies suggest a positive role of *ENO1* in tumour cell invasion.

In addition to being responsible for neoplastic transformation, more and more evidence show that oncogenes also build up an inflammatory microenvironment for tumour progression.[31] For instance, several cytokines were up-regulated in RAS oncogene-driven tumourigenesis.[32] Consistent with this notion, the expression of chemokines and their cognate receptors in cancer cells play important roles in tumour growth and metastatic spread.[33] *CCL20*, alternatively named liver and activation-regulated chemokine (LARC), macrophage inflammatory protein-3α (MIP-3α) or Exodus-1, was the most expressed chemokine induced by ectopic *ENO1* (Table S3). CCR6 is the only cognate receptor for CCL20.[34] We found the highest mRNA expression of CCR6 amongst ten CCR genes in the parental CAL27 cells. Ectopic *ENO1*, however, had no effect on CCR6 expression in our array data (data not shown). CCL20–CCR6 participates both in the chemoattraction of inflammatory cells and in the carcinogenesis of liver and pancreas.[25,35] This ligand–receptor pair also promotes the invasion of pancreatic and nasopharyngeal carcinoma cells.[26,28] Reducing *CCL20* expression by specific shRNA significantly attenuated the transforming and invasive ability of ENO1-expressing oral cancer cells. Together, CCL20 chemokine is one downstream effecter participating in the transforming and invasive actions of *ENO1* gene product. More studies are, however, needed to address if the expression of *CCL20* is directly or indirectly mediated by *ENO1* gene and if this chemokine exerts its autocrine or paracrine actions in head and neck cancer.

In summary, tumour cells increase nutrient uptake by glycolysis to support the enhanced metabolism for their unrestricted growth during tumourigenesis. Interfering with the glycolytic phentoype frequently amplified in cancer cells is now attracting great interest as a possible therapeutic means to limit energy production in a tumour cell-specific fashion. *ENO1* overexpression promoted cell proliferation, migration, invasion and tumourigenesis. Knocking down the expression of *ENO1* or *CCL20* significantly reduced the transforming and invasive ability of *ENO1*-overexpressing tumour cells. The *ENO1* gene, a prognostic factor, and *CCL20* may thus be used as therapeutic targets for treating *ENO1*-overexpressing head and neck cancer.

Role of funding sources

Both National Science Council and Department of Health in Taiwan are governmental funding agencies with missions to promote science and health awareness in general public.

Conflict of interest statement

None declared.

Acknowledgements

Chen, Chiu-Chen at the Center of Excellence for Clinical Trial and Research in Oncology Specialty did statistical analysis of clinical data. ShRNA reagents were from the National RNAi Core Facility at Academia Sinica in Taiwan. This work was supported by National Science Council (NSC96-2628-B006-012 and NSC96-2320-B006-046) and Department of Health (DOH-TD-B-111-004) in Taiwan.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.03.018.

REFERENCES

1. Jin S, DiPaola RS, Mathew R, White E. Metabolic catastrophe as a means to cancer cell death. *J Cell Sci* 2007;**120**(Pt 3):379–83.
2. Mankoff DA, Dunnwald LK, Partridge SC, Specht JM. Blood flow-metabolism mismatch: good for the tumor, bad for the patient. *Clin Cancer Res* 2009;**15**(17):5294–6.
3. Brahimi-Horn C, Pouyssegur J. The role of the hypoxia-inducible factor in tumor metabolism growth and invasion. *Bull Cancer* 2006;**93**(8):E73–80.
4. Altenberg B, Greulich KO. Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes. *Genomics* 2004;**84**(6):1014–20.
5. Kim JW, Dang CV. Multifaceted roles of glycolytic enzymes. *Trends Biochem Sci* 2005;**30**(3):142–50.
6. Pancholi V. Multifunctional alpha-enolase: its role in diseases. *Cell Mol Life Sci* 2001;**58**(7):902–20.
7. Miles LA, Dahlberg CM, Plescia J, et al. Role of cell-surface lysines in plasminogen binding to cells: identification of alpha-enolase as a candidate plasminogen receptor. *Biochemistry* 1991;**30**(6):1682–91.
8. Dano K, Behrendt N, Hoyer-Hansen G, et al. Plasminogen activation and cancer. *Thromb Haemost* 2005;**93**(4):676–81.
9. Wygrecka M, Marsh LM, Morty RE, et al. Enolase-1 promotes plasminogen-mediated recruitment of monocytes to the acutely inflamed lung. *Blood* 2009;**113**(22):5588–98 [Epub 30th January 2009].
10. Liu K-J, Shih N-Y. The role of enolase in tissue invasion and metastasis of pathogens and tumor cells. *J Cancer Mol* 2007;**3**(2):45–8.
11. Hamaguchi T, Iizuka N, Tsunedomi R, et al. Glycolysis module activated by hypoxia-inducible factor 1alpha is related to the aggressive phenotype of hepatocellular carcinoma. *Int J Oncol* 2008;**33**(4):725–31.
12. Racz A, Brass N, Hofer M, et al. Gene amplification at chromosome 1pter-p33 including the genes PAX7 and ENO1 in squamous cell lung carcinoma. *Int J Oncol* 2000;**17**(1):67–73.
13. Semenza GL, Jiang BH, Leung SW, et al. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J Biol Chem* 1996;**271**(51):32529–37.
14. Osthus RC, Shim H, Kim S, et al. Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. *J Biol Chem* 2000;**275**(29):21797–800.
15. Chang YS, Wu W, Walsh G, Hong WK, Mao L. Enolase-alpha is frequently down-regulated in non-small cell lung cancer and predicts aggressive biological behavior. *Clin Cancer Res* 2003;**9**(10 Pt 1):3641–4.
16. Ejeskar K, Krona C, Caren H, et al. Introduction of *in vitro* transcribed ENO1 mRNA into neuroblastoma cells induces cell death. *BMC Cancer* 2005;**5**:161.
17. Chang GC, Liu KJ, Hsieh CL, et al. Identification of alpha-enolase as an autoantigen in lung cancer: its overexpression is associated with clinical outcomes. *Clin Cancer Res* 2006;**12**(19):5746–54.
18. He P, Naka T, Serada S, et al. Proteomics-based identification of alpha-enolase as a tumor antigen in non-small lung cancer. *Cancer Sci* 2007;**98**(8):1234–40.
19. Wu W, Tang X, Hu W, et al. Identification and validation of metastasis-associated proteins in head and neck cancer cell lines by two-dimensional electrophoresis and mass spectrometry. *Clin Exp Metastasis* 2002;**19**(4):319–26.
20. Deshpande AM, Wong DT. Molecular mechanisms of head and neck cancer. *Expert Rev Anticancer Ther* 2008;**8**(5):799–809.
21. Tsantoulis PK, Kastrinakis NG, Tourvas AD, Laskaris G, Gorgoulis VG. Advances in the biology of oral cancer. *Oral Oncol* 2007;**43**(6):523–34.
22. Ito S, Honma T, Ishida K, et al. Differential expression of the human alpha-enolase gene in oral epithelium and squamous cell carcinoma. *Cancer Sci* 2007;**98**(4):499–505.
23. Tsai WC, Tsai ST, Jin YT, Wu LW. Cyclooxygenase-2 is involved in S100A2-mediated tumor suppression in squamous cell carcinoma. *Mol Cancer Res* 2006;**4**(8):539–47.
24. Abiko Y, Nishimura M, Kusano K, et al. Expression of MIP-3alpha/CCL20, a macrophage inflammatory protein in oral squamous cell carcinoma. *Arch Oral Biol* 2003;**48**(2):171–5.
25. Kleeff J, Kusama T, Rossi DL, et al. Detection and localization of Mip-3alpha/LARC/Exodus, a macrophage proinflammatory chemokine, and its CCR6 receptor in human pancreatic cancer. *Int J Cancer* 1999;**81**(4):650–7.
26. Campbell AS, Albo D, Kimsey TF, White SL, Wang TN. Macrophage inflammatory protein-3alpha promotes pancreatic cancer cell invasion. *J Surg Res* 2005;**123**(1):96–101.
27. Fujii H, Itoh Y, Yamaguchi K, et al. Chemokine CCL20 enhances the growth of HuH7 cells via phosphorylation of p44/42 MAPK *in vitro*. *Biochem Biophys Res Commun* 2004;**322**(3):1052–8.
28. Chang KP, Hao SP, Chang JH, et al. Macrophage inflammatory protein-3alpha is a novel serum marker for nasopharyngeal carcinoma detection and prediction of treatment outcomes. *Clin Cancer Res* 2008;**14**(21):6979–87.
29. Subramanian A, Miller DM. Structural analysis of alpha-enolase. Mapping the functional domains involved in down-regulation of the c-myc protooncogene. *J Biol Chem* 2000;**275**(8):5958–65.
30. Takashima M, Kuramitsu Y, Yokoyama Y, et al. Overexpression of alpha enolase in hepatitis C virus-related hepatocellular carcinoma: association with tumor progression as determined by proteomic analysis. *Proteomics* 2005;**5**(6):1686–92.
31. Borrello MG, Degl'Innocenti D, Pierotti MA. Inflammation and cancer: the oncogene-driven connection. *Cancer Lett* 2008;**267**(2):262–70.
32. Ancrile BB, O'Hayer KM, Counter CM. Oncogenic ras-induced expression of cytokines: a new target of anti-cancer therapeutics. *Mol Interv* 2008;**8**(1):22–7.
33. Singh S, Sadanandam A, Singh RK. Chemokines in tumor angiogenesis and metastasis. *Cancer Metastasis Rev* 2007; **26**(3–4):453–67.
34. Schutyser E, Struyf S, Van Damme J. The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev* 2003;**14**(5):409–26.
35. Rubie C, Frick VO, Wagner M, et al. Enhanced expression and clinical significance of CC-chemokine MIP-3 alpha in hepatocellular carcinoma. *Scand J Immunol* 2006;**63**(6):468–77.