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GDF15 is a potential predictive biomarker for TPF induction chemotherapy and promotes tumorigenesis and progression in oral squamous cell carcinoma

C. Z. Yang[1,†], J. Ma[1,†], D. W. Zhu[1], Y. Liu[1], B. Montgomery[2], L. Z. Wang[3], J. Li[3], Z. Y. Zhang[1], C. P. Zhang[1] & L. P. Zhong[1*]

[1]*Department of Oral and Maxillofacial-Head and Neck Oncology, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China;* [2]*University of Missouri, Kansas City School of Medicine, Kansas City, USA;* [3]*Department of Oral Pathology, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China*

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Background: Randomized trials have not shown major survival benefits when induction chemotherapy plus standard therapy is compared with standard therapy alone in patients with oral squamous cell carcinoma (OSCC). Induction chemotherapy is likely to be effective for biologically distinct subgroups and biomarker development may lead to identification of patients whose tumors are likely to respond to a particular treatment.

Patients and methods: We evaluated immunohistochemical staining for GDF15 in pretreatment biopsy specimens of 230 of 256 OSCC patients who were treated in a prospective, randomized, phase III trial on induction chemotherapy including docetaxel, cisplatin and 5-fluorouracil (TPF). Relationship between GDF15 intervention and cell proliferation, migration, invasion, colony formation and tumorigenicity was analyzed using *in vitro* and *in vivo* OSCC models.

Results: Low GDF15 expression predicted a better survival in OSCC patients, especially overall survival [$P = 0.049$, hazard ratio (HR) = 0.597] and distant metastasis-free survival (DMFS; $P = 0.031$, HR = 0.562). cN+ patients with low GDF15 expression benefitted from induction TPF in overall survival ($P = 0.039$, HR = 0.247) and DMFS ($P = 0.039$, HR = 0.247), cN− patients with high GDF15 expression benefitted from induction TPF in overall survival ($P = 0.019$, HR = 0.231), disease-free survival ($P = 0.011$, HR = 0.281), locoregional recurrence-free survival ($P = 0.035$, HR = 0.347) and DMFS ($P = 0.009$, HR = 0.197). Decreased GDF15 expression in OSCC lines significantly inhibited cell proliferation, migration, invasion, colony formation and tumorigenesis through increased phosphorylation of AKT and ERK1/2 ($P < 0.05$). Likewise, overexpression of GDF15 significantly promoted cell proliferation, migration, invasion and colony formation through decreased phosphorylation of AKT and ERK1/2 ($P < 0.05$).

Conclusions: GDF15 expression can be used as a prognostic biomarker for OSCC, and as a predictive biomarker for benefitting from TPF induction chemotherapy. GDF15 promotes tumorigenesis and progression through phosphorylation of AKT and ERK1/2 in OSCC. The clinical trial in this study was registered with www.ClinicalTrials.gov (NCT01542931).

Key words: growth differentiation factor 15, oral squamous cell carcinoma, induction chemotherapy, prognosis, tumorigenesis

introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cancer by incidence worldwide [1]. Oral squamous cell carcinoma (OSCC), a subset of this disease, has a poor clinical outcome with a 5-year survival rate of only 50%–60% [1, 2]. Currently, the treatment strategy for patients with locally advanced and resectable OSCC is radical surgery followed by postoperative radiation or chemoradiation, depending on the presence of high-risk features in the surgical specimen [3]. Clinically, only staging and pathologic differentiation grade are used to predict the prognosis of OSCC patients [4]. Therefore, it is critical to understand the biological basis of OSCC and develop novel biomarkers that can help predict the prognosis or the response to a particular treatment strategy, such as induction chemotherapy.

Induction chemotherapy is regarded as an effective way to reduce locally advanced or aggressive cancers to improve the chance of eradication of locoregional lesions by radical surgery and/or radiation/chemoradiation. However, it is still unknown whether induction chemotherapy protocol of docetaxel,

Correspondence to: Dr Lai-ping Zhong, Department of Oral and Maxillofacial-Head and Neck Oncology, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine; No. 639 Zhizaoju Road, Shanghai 200011, China. Tel: +86-21-23271699-5160; Fax: +86-21-63136856; E-mail: zhonglp@hotmail.com

†C. Z. Yang and J. Ma contributed equally to this paper.

cisplatin and 5-fluorouracil (TPF) improves outcomes when given before surgery in the patients with locally advanced HNSCC, especially OSCC [5]. To address the role of TPF induction chemotherapy in OSCC treated with surgery and radiation, we previously conducted a randomized phase III trial of TPF induction chemotherapy followed by surgery and radiation versus surgery and radiation in patients with locally advanced and resectable OSCC [6]. Although we failed to demonstrate a survival advantage for TPF induction chemotherapy in the overall study population, it is possible that TPF induction chemotherapy might improve outcomes in a molecularly defined subset of patients. Correlative studies from the aforementioned randomized trials could assist in identifying candidate biomarkers predictive of benefit from TPF induction chemotherapy.

Growth differentiation factor 15 (GDF15) is a divergent member of the TGF-β superfamily. It plays multiple roles in various pathologies, including inflammation, cancer, cardiovascular diseases and obesity [7, 8]. In cancer, GDF15 has been reported to have both tumorigenic and anti-tumorigenic activities [8, 9]. Though GDF15's role in tumorigenesis is probably not universal in all cancers, overexpression of GDF15 has been reported in OSCC patients [10, 11]. Unfortunately, neither the clinical usefulness of GDF15 as a potential prognostic or predictive biomarker for TPF induction chemotherapy, nor the mechanism of GDF15 on tumorigenesis and progression in OSCC have been described in the literature.

In this study, we found that GDF15 might be used as a prognostic and predictive biomarker for TPF induction chemotherapy in locally advanced OSCC; GDF15 could promote tumorigenesis and progression of OSCC through phosphorylation of AKT and ERK1/2.

patients and methods

patients

From March 2008 to December 2010, 256 patients with primary and locally advanced OSCC from a prospective, randomized, phase III trial at Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine were enrolled into this study. The hypothesis of trial was that TPF induction chemotherapy administered before surgery and postoperative radiation improves survival in patients with locally advanced OSCC (trial registration ID: NCT01542931) [6]. Pretreatment formalin-fixed and paraffin-embedded biopsy samples were collected. If pretreatment biopsy was unavailable in those treated with surgery and postoperative radiation alone, resected surgical samples were collected for examination.

immunohistochemistry

Briefly, sections were incubated with the rabbit polyclonal antibody against GDF15 (1 : 100) (Abcam, UK) and visualized using 3,3′-diaminobenzidine detection kit (Dako Cytomation, Denmark). GDF15 positive grade was determined based on the Immuno-Reactive-Score (IRS) system, GDF15 expression was low when IRS = 0–3 and high when IRS = 4–12 [12] (detail in the supplementary Methods, available at *Annals of Oncology* online).

in vitro and *in vivo* experiments assays

Cell cultures, GDF15 RNA interference and gene transfection, real-time PCR, western blot and antibodies, cell cycle and apoptosis, cell growth, migration and invasion, colony formation and subcutaneous tumorigenesis in NOD/SCID mice were provided in the supplementary Methods, available at *Annals of Oncology* online.

statistical analysis

After treatment, patients were monitored every 3 months in the first 2 years, every 6 months in the subsequent 3–5 years and once a year thereafter until death or data censoring. Overall survival (OS) was calculated from the date of randomization to the date of death. Disease-free survival (DFS), locoregional recurrence-free survival (LRFS) and distant metastasis-free survival (DMFS) were calculated from the date of randomization to recurrence, locoregional recurrence, distant metastasis or death from any cause, respectively. The survival analysis was conducted using the Kaplan–Meier method and log-rank test. Intention-to-treat principle was applied for efficacy analysis.

All hypothesis-generating tests were two-sided at a significance level of 0.05. Data were analyzed with the statistical software SPSS13.0 for Windows (SPSS, Inc., Chicago, USA).

results

patient characteristics and treatment outcomes

Two hundred fifty-six patients were enrolled in this trial, with 128 patients in each arm. Pretreatment biopsy samples were gathered from 230 of these patients (126 patients in the control arm, 104 patients in the experimental arm). Pretreatment GDF15 expression was assessed in the tumors. The distribution of baseline characteristics in the subset of patients that had biomarker evaluation was similar to the distribution in the entire trial population (supplementary Table S1, available at *Annals of Oncology* online). Patients were followed until June 2013; the median follow-up time was 48 months among the censored patients.

Although patients in the experimental arm had a slightly better OS, DFS, LRFS and DMFS compared with those in the control arm, the difference was not significant (supplementary Figure S1, available at *Annals of Oncology* online). The 3-year OS, DFS, LRFS and DMFS in the control arm was 63.1%, 55.4%, 59.2% and 60.9%, respectively; that in the experimental arm was 68.9%, 61.5%, 61.3% and 68.9%, respectively. The locoregional recurrence rate was 38.9% and the distant metastasis rate was 10.3% in the control arm, which was 30.8% and 5.8% in the experimental arm.

GDF15 expression in OSCC patients

In the 230 patients, 68 samples (37 in the control arm and 31 in the experimental arm) were found to have low GDF15 expression, including 33 negative and 35 weak positive; 162 samples (89 in the control arm and 73 in the experimental arm) were found to have high GDF15 expression (supplementary Figure S2A–D, available at *Annals of Oncology* online). There was an equal distribution of GDF15 expression between the two arms (χ^2 test = 0.005, $P = 0.942$). No significant difference of proportion of GDF15 expression was found according to the baseline characteristics with exception of alcohol use (supplementary Table S1, available at *Annals of Oncology* online). The proportion of patients with high GDF15 expression was higher amongst patients with negative alcohol use (107 of 142) compared with those with positive alcohol use (55 of 88).

GDF15 expression as a prognostic biomarker

The patients with low GDF15 expression had a better survival, especially OS [$P = 0.049$, hazard ratio (HR) = 0.597] and DMFS ($P = 0.031$, HR = 0.562) (Figure 1). In the patients with low GDF15 expression, the 3-year OS, DFS, LRFS and DMFS was 76.4%, 65.8%, 67.3% and 76.4%, respectively, the locoregional recurrence rate was 27.9% and the distant metastasis rate was 5.9%. In the patients with high GDF15 expression, the 3-year OS, DFS, LRFS and DMFS was 61.4%, 54.9%, 57.2% and 59.7%, respectively, the locoregional recurrence rate was 38.3% and the distant metastasis rate was 9.3%. Univariate Cox model was used to analyze the impact of baseline characteristics on the time-to-event end points. GDF15 expression (low versus high), lymph node status (cN0-1 versus cN2, or cN0 versus cN1-2) and clinical stage (stage III versus stage IVA) were risk factors on OS, DFS, LRFS or DMFS. Multivariate Cox model analysis was carried out using the risk factors of GDF15 expression and clinical stage, while lymph node status (cN0-1 versus cN2 or cN0 versus cN1-2) was not used because of the direct correlation between clinical stage and lymph node status. Both the clinical stage ($P = 0.001$) and GDF15 expression ($P = 0.009$) were independent risk factors. When pathologic differentiation grade and alcohol use were used in the multivariate Cox model analysis, only the clinical stage ($P = 0.001$) and GDF15 expression ($P = 0.006$) were independent risk factors.

GDF15 expression as a predictive biomarker of benefitting from TPF induction chemotherapy

To explore whether GDF15 expression could predict benefit from TPF induction chemotherapy, we analyzed the interaction among GDF15 expression, treatment and survival outcome. There were no significant differences in OS, DFS, LRFS or DMFS between the experimental and control arms in patients with low or high GDF15 expression (supplementary Table S2, available at *Annals of Oncology* online). Subset analysis based on clinical characteristics showed that cN− patients with high GDF15 expression benefitted from TPF induction chemotherapy in OS ($P = 0.019$, HR = 0.231), DFS ($P = 0.011$, HR = 0.281), LRFS ($P = 0.035$, HR = 0.347) and DMFS ($P = 0.009$, HR = 0.197); cN+ patients with low GDF15 expression benefitted from TPF induction chemotherapy in OS ($P = 0.039$, HR = 0.247) and DMFS ($P = 0.039$, HR = 0.247) (supplementary Figure S3, available at *Annals of Oncology* online).

GDF15 expression in OSCC cell lines

Increased GDF15 protein expression was found in five OSCC cell lines of HB96, HN6, HN30, CAL27 and SCC4 compared with the immortalized cell line of human immortalized oral epithelial cell (HIOEC) (supplementary Figure S2E and F, available at *Annals of Oncology* online).

downregulation of GDF15 expression inhibits cell proliferation and increases apoptosis

Both sequences of shGDF15-1 and shGDF15-2 were used to silence GDF15 expression in the HB96 and HN30 lines. After cells transfected successfully with shGDF15-1 or shGDF15-2 (Supplementary Figure S2G and H, available at *Annals of Oncology* online), a significant decrease in cell proliferation was found in both HB96 and HN30 cells compared with the controls (Figure 2A–D). Using cell cycle analysis, the proportion of cells in the S phase was significantly lower in the HB96 and HN30 cells transfected with shGDF15-1 or shGDF15-2 than the controls (Figure 2E). Using flow cytometric analysis, a significant increase in the percentage of cell apoptosis was found in the HB96 and HN30 cells transfected with shGDF15-1 or shGDF15-2 compared with the controls (Figure 2F).

downregulation of GDF15 expression inhibits cell migration, invasion, colony formation and tumorigenesis

Using wound-healing assay, the HB96 and HN30 cells transfected with shGDF15-1 or shGDF15-2 showed a significantly slower healing speed than the controls (supplementary Figure S4, available at *Annals of Oncology* online). After cells seeded on Matrigel-coated membrane, there were significantly less invasive cells in the HB96 and HN30 cells transfected with shGDF15-1 or shGDF15-2 compared with the controls. The colony formation was significantly inhibited in the HB96 and HN30 cells transfected with shGDF15-1 or shGDF15-2 compared with the controls. To test the tumorigenesis *in vivo*, the HN30-shGDF15-1 and HN30-scramble-1 cells were inoculated subcutaneously in the NOD/SCID mice. The tumor size in the HN30-shGDF15-1 group was significantly smaller than the HN30-scramble-1 group, and the tumor weight in the HN30-shGDF15-1 group was also significantly less than the HN30-scramble-1 group. There was no tumorigenesis in seven out of ten sites in the HN30-shGDF15-1 group; however, tumorigenesis was found in all ten sites in the HN30-scramble-1 group (Figure 3).

overexpression of GDF15 increases cell proliferation, migration, invasion and colony formation

After cells transfected with lentivirus vector containing the whole GDF15 gene (EGFP-IRES-PURO-GDF15) or empty control (supplementary Figure S2I and J, available at *Annals of Oncology* online), the HIOEC and HB96 cells with GDF15 overexpression grew significantly faster than those transfected with empty control. The proportion of S phase cells was higher in the HIOEC and HB96 cells with GDF15 overexpression than those transfected with empty control. The HIOEC and HB96 cells with GDF15 overexpression also had a faster healing speed than those transfected with empty control. The numbers of invasive cells and colony formation were higher in the HIOEC and HB96 cells with GDF15 overexpression than the controls (supplementary Figure S5, available at *Annals of Oncology* online). Unfortunately, the difference in number of invasive cells and colony formation was not significant between the HB96 cells transfected with and without GDF15 overexpression.

GDF15 expression correlates with phosphorylation of AKT and ERK1/2

In the HB96 and HN30 cells transfected with shGDF15-1 or shGDF15-2, a significantly lower level of AKT and ERK 1/2 phosphorylation accompanied the decrease in GDF15 expression, when compared with the cells transfected with scramble

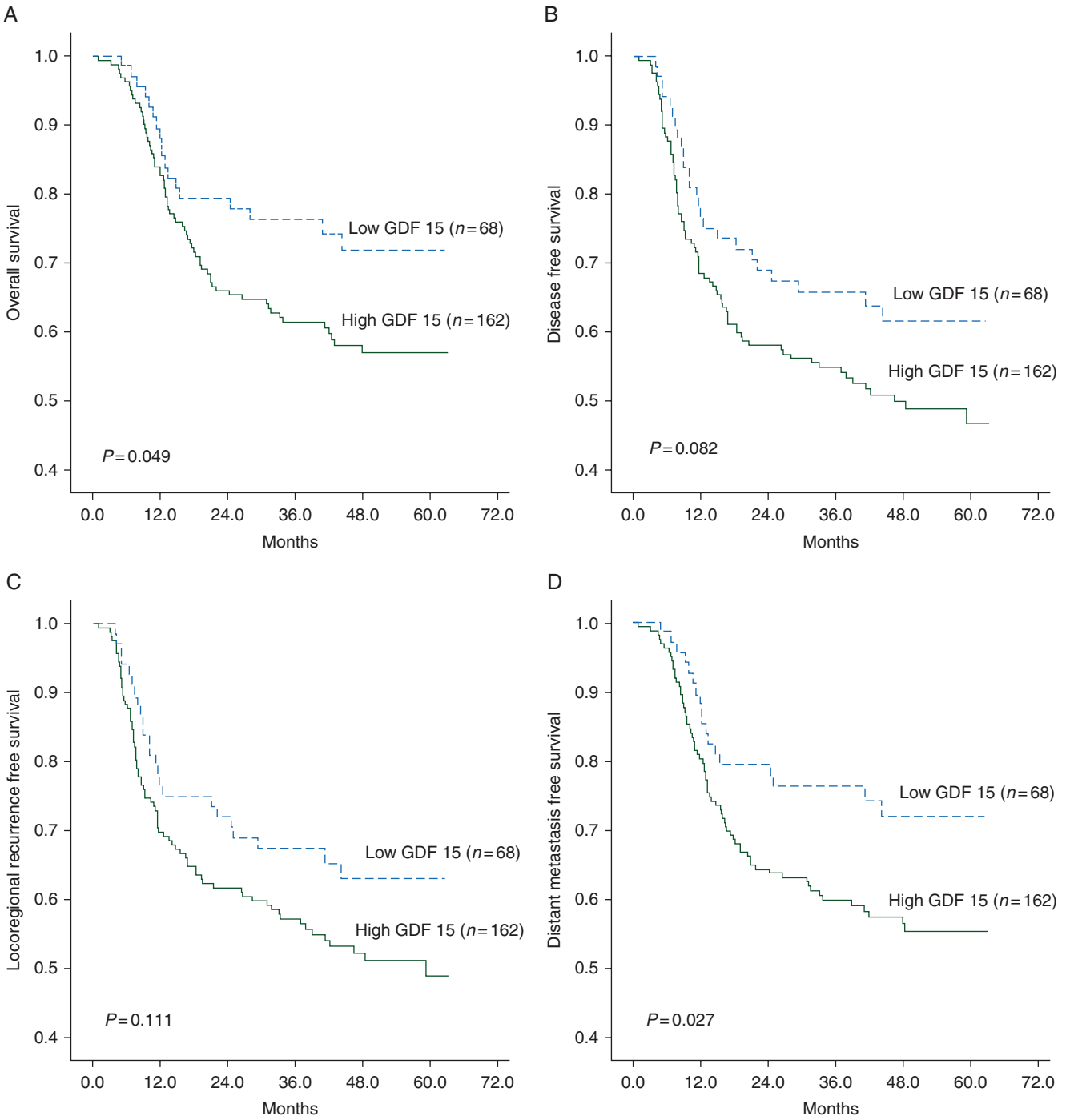

Figure 1. Overall survival, disease-free survival, locoregional recurrence-free survival and distant metastasis-free survival in the patients with low and high GDF15 expression. Patients with low GDF15 expression had a significantly better overall survival (A) and distant metastasis-free survival (D) compared with those with high GDF15 expression; a trend of patients with low GDF15 expression had a better disease-free survival (B) and locoregional recurrence-free survival (C) compared with those with high GDF15 expression.

sequences. At the same time, the level of cleaved PARP and BAX increased in both HB96 and HN30 cells transfected with shGDF15-1 or shGDF15-2. On the other hand, increased level of AKT and ERK1/2 phosphorylation was found in the HIOEC and HB96 cells with GDF15 overexpression compared with those transfected with empty control (Supplementary Figure S6, available at *Annals of Oncology* online).

discussion

In this study, we found that GDF15 expression could be used as a prognostic and predictive biomarker for OSCC. The patients with low GDF15 expression had increased survival in comparison with patients with high GDF15 expression, especially in OS and DMFS. cN+ patients with low GDF15 expression and

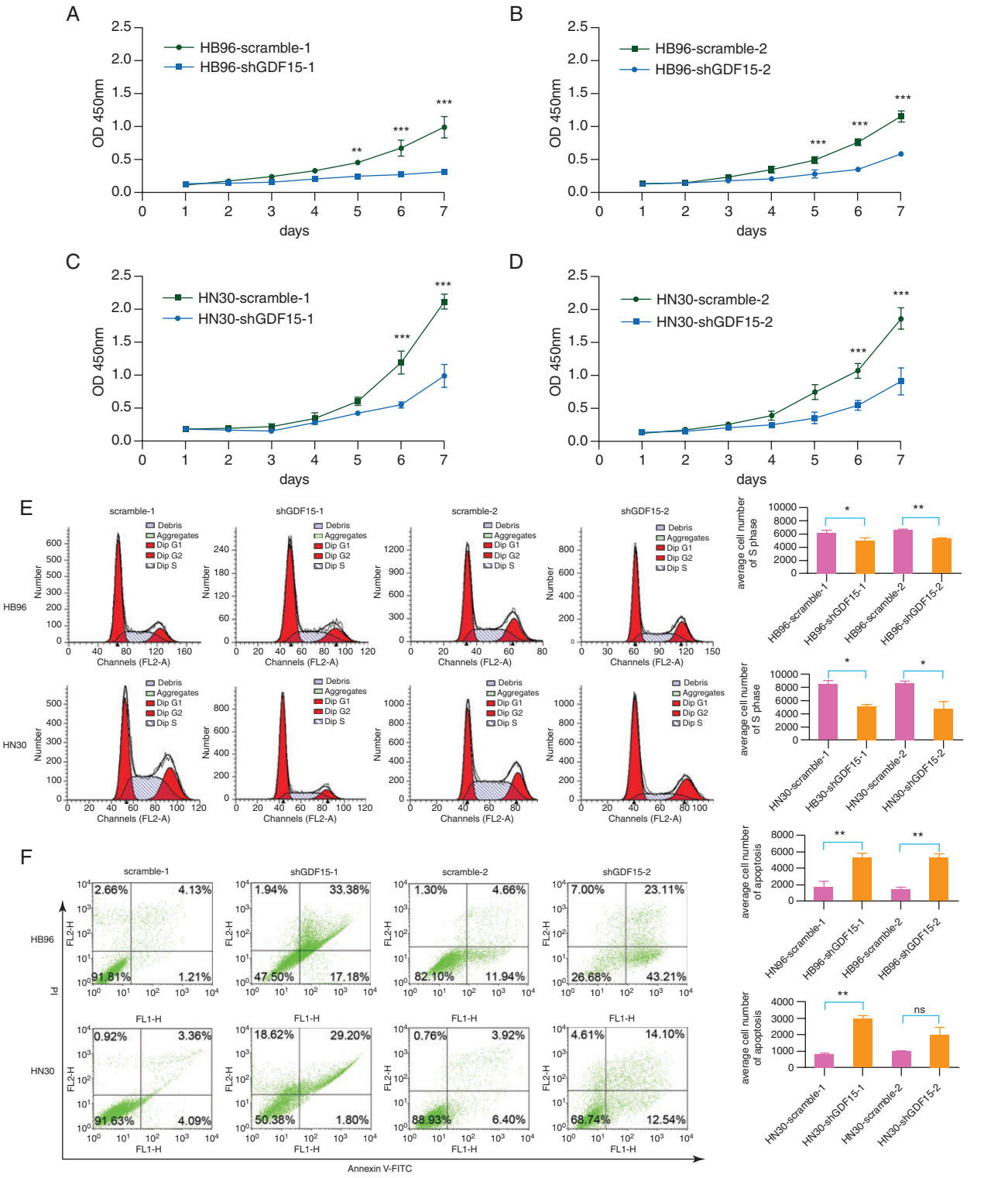

Figure 2. Downregulation of GDF15 expression inhibited cell proliferation and promoted cell apoptosis in the HB96 and HN30 cells. (A) HB96 cells transfected with shGDF15-1 grew more slowly than those transfected with scramble-1 sequence; (B) HB96 cells transfected with shGDF15-2 grew more slowly than those transfected with scramble-2 sequence; (C) HN30 cells transfected with shGDF15-1 grew more slowly than those transfected with scramble-1 sequence; (D) HN30 cells transfected with shGDF15-2 grew more slowly than those transfected with scramble-2 sequence. (E) Cell cycle analysis showed that the population of HB96 and HN30 cells transfected with shGDF15-1 and shGDF15-2 displayed remarkably decreased time in the S phase compared with the cells transfected with scramble-1 and scramble-2, respectively. (F) Flow cytometric analysis showed that the percentage of cell apoptosis in the HB96 and HN30 cells transfected with shGDF15-1 and shGDF15-2 was significantly increased compared with the cells transfected with scramble-1 and scramble-2, respectively. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, ns: $P = 0.081$.

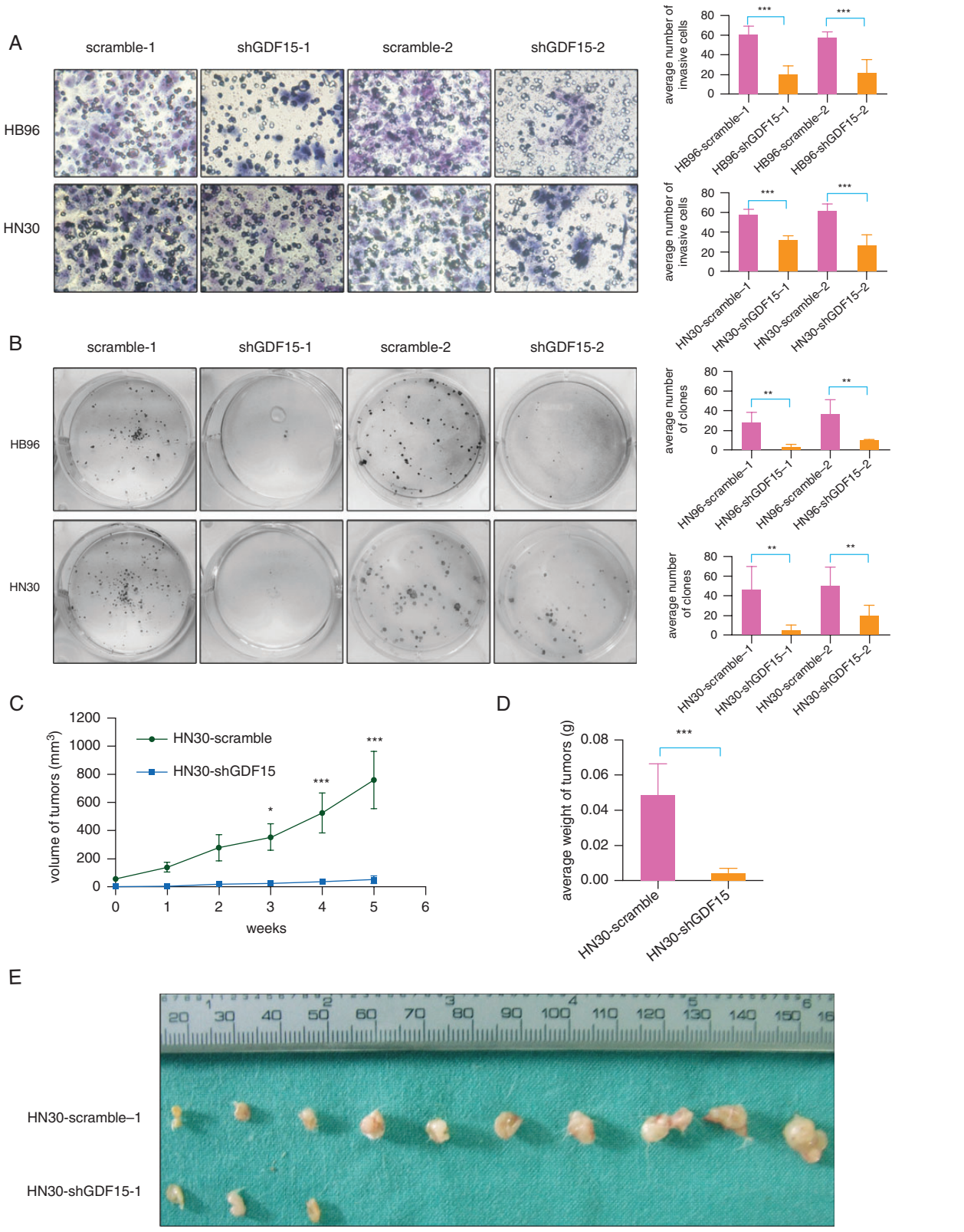

Figure 3. Downregulation of GDF15 expression inhibited cell invasion, colony formation and tumorigenicity in the HB96 and HN30 cells. (A) Using cell invasion assay, the number of invasive cells was less in the HB96 and HN30 cells transfected with shGDF15-1 or shGDF15-2, compared with those transfected with scramble sequences. (B) Using colony formation assay, the colony formation was significantly inhibited in the HB96 and HN30 cells transfected with shGDF15-1 or shGDF15-2 compared with those transfected with scramble sequences. (C) Using *in vivo* subcutaneous tumorigenesis in the NOD/SCID mice, the growth of tumor was slower in the HN30 cells transfected with shGDF15-1 compared with those transfected with scramble-1. (D) The weight of tumor was also lighter in the HN30 cells transfected with shGDF15-1 compared with those transfected with scramble-1. (E) In the HN30-scramble-1 group, tumorigenesis was found in all injection sites, and in the HN30-shGDF15-1 group, tumorigenesis was found in 3 of 10 injection sites. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

cN− patients with high GDF15 expression could benefit from TPF induction chemotherapy.

We also found that GDF15 could promote the tumorigenesis and progression of OSCC through AKT and ERK1/2 phosphorylation. After downregulating GDF15 expression in OSCC lines, the phosphorylation of AKT and ERK1/2 was decreased, and the cell proliferation, migration, invasion, colony formation and tumorigenicity were inhibited. Flow cytometric analysis and identification of cleaved PARP and BAX confirmed that cell apoptosis was prompted. Upregulation of GDF15 expression in HIOEC and HB96 lines led to increased levels of AKT and ERK1/2 phosphorylation, and increased cell proliferation, migration, invasion and colony formation.

GDF15 mediates various physiological and pathology functions including the development of the embryo, regulation of cellular stress and immune response, tissue repair and tumor progress. Numerous studies indicate that enhanced GDF15 levels contribute to cancer progression and tumor-associated weight loss. Notably, cancer progression and increased GDF15 levels have been reported for brain, melanoma, lung, thyroid, gastrointestinal, colorectal, pancreatic, prostate, breast and cervical epithelial cancers [8, 9].

Elevated GDF15 expression could be used as a potential prognostic biomarker in several cancers such as melanoma, prostate, gastric, colorectal, pancreas, endometrial, ovarian and head and neck cancers [10, 13–16]. In the present study, OSCC patients with low GDF15 expression have a better prognosis than those with high GDF15 expression. Potential usefulness of GDF15 as a predictive biomarker for chemotherapy or induction chemotherapy has been noted in ovarian and prostate cancers [17, 18]. And to our knowledge, this is the first time that GDF15 is assessed in a cohort of patients prospectively followed within the context of a randomized induction chemotherapy trial. We found that cN+ patients with low GDF15 expression and cN− patients with high GDF15 expression could benefit from TPF induction chemotherapy in OSCC. However, this requires further validation in other datasets. One could envision a personalized treatment scenario in which OSCC patients with cN+/low GDF15 expression and cN−/high GDF15 expression receive TPF induction chemotherapy before surgery while those patients with cN−/high GDF15 expression and cN+/low GDF15 expression are treated with surgery upfront, in order to avoid the toxicity from chemotherapeutic agents and to avoid the delay of definitive treatment.

The exact mechanism of GDF15 in tumorigenesis and progression is not well understood. There are still controversial results of GDF15's effect on tumorigenesis and progression *in vitro* and *in vivo*. In HCT-166 colorectal cancer cell line and MDA-MB-468 and MCF-7 breast cancer cell lines, overexpression of GDF15 results in decreased cell viability and tumorigenicity [19, 20]. However, other studies have reported that overexpression of GDF15 promotes tumorigenesis and progression in cancer cell lines [21–23]. In our study, we demonstrated that decreased GDF15 expression could inhibit cell proliferation, migration, invasion, colony formation and tumorigenesis, and promote cell apoptosis in OSCC; increased GDF15 expression could promote cell proliferation, migration, invasion and colony formation. Therefore, overexpression of GDF15 plays an important role in tumorigenesis and progression in OSCC.

GDF15 has been reported as a target gene of p53, because there are two binding sites for p53 in the GDF15 promoter [24]. Furthermore, GDF15 is also involved in several signaling pathways, including the focal adhesion kinase pathway, ERK1/2 pathway, PI3K/AKT pathway, TGF-β RII pathway, among others [9, 25]. In our present study, we provided the evidence for the first time that in OSCC, GDF15 expression promotes tumorigenesis and progression through phosphorylation of AKT and ERK1/2. When shRNA against GDF15 was transfected into OSCC lines, decreased expression of GDF15 was accompanied by decreased phosphorylation of AKT and ERK1/2, and decreased cell proliferation, migration, invasion, colony formation and tumorigenicity of OSCC lines. When whole GDF15 gene was transfected into immortalized oral mucosal cell lines and OSCC lines, increased GDF15 expression was accompanied by increased phosphorylation of AKT and ERK1/2, and increased cell proliferation, migration, invasion and colony formation. However, further molecular studies are encouraged to clear the detail mechanism of GDF15 in the signaling pathways.

In conclusion, our results reveal that GDF15 expression can be used as a prognostic biomarker for OSCC and as a predictive biomarker for benefitting from TPF induction chemotherapy. GDF15 promotes tumorigenesis and progression of OSCC through phosphorylation of AKT and ERK1/2. Further trials are encouraged to confirm the GDF15 expression as predictive biomarker in OSCC.

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disclosure

The authors have declared no conflicts of interest.

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Outcome of patients with sarcoma and other mesenchymal tumours participating in phase I trials: a subset analysis of a European Phase I database

P. A. Cassier[1,2,3*], V. Polivka[4], I. Judson[1,2], J. -C. Soria[5], N. Penel[6], S. Marsoni[7], J. Verweij[8], J. H. Schellens[9], R. Morales-Barrera[10], P. Schöffski[11,12], E. E. Voest[13], C. Gomez-Roca[5], T. R. J. Evans[14], R. Plummer[15], E. Gallerani[16], S. B. Kaye[1,2] & D. Olmos[1,2]

[1]Drug Development Unit, The Royal Marsden National Health Service Foundation Trust, Sutton; [2]The Institute of Cancer Research, Sutton, UK; Departments of [3]Medical Oncology; [4]Biostatistics, Centre Léon Bérard, Lyon; [5]Department of Medicine, Institut Gustave Roussy, Villejuif; [6]Department of General Cancer, Centre Oscar Lambret, Lille, France; [7]Southern Europe New Drug Organization Foundation, Milan, Italy; [8]Erasmus University Medical Center, Cancer Institute, Rotterdam; [9]The Netherlands Cancer Institute, Amsterdam, The Netherlands; [10]Research Unit for Molecular Therapy of Cancer, Vall d'Hebron University Hospital, Vall d'Hebron Institute of Oncology (VHIO), Universitat Autònoma de Barcelona, Barcelona, Spain; [11]Department of General Medical Oncology, University Hospitals Leuven, Leuven; [12]Laboratory of Experimental Oncology, KU Leuven, Leuven, Belgium; [13]University Medical Center Utrecht, Utrecht, The Netherlands; [14]The Beatson West of UK Cancer Centre, Glasgow; [15]Northern Centre for Cancer Care, Freeman Hospital, Newcastle upon Tyne, UK; [16]Department of Oncology, Istituto Oncologico Della Svizzera Italiana, Bellinzona, Switzerland

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Background: Although sarcomas account for only 1% of all solid tumours, patients with sarcomas comprise a larger proportion of patients entering phase I trials, due to the limited number of registered or active drugs for these diseases. To

*Correspondence to: Dr Philippe A. Cassier, Department of Médical Oncology, Centre Léon Bérard, 28 rue Laennec, 69008 Lyon, France. Tel: +33-4-26-55-68-33; E-mail: cassierp@hotmail.com