

Transglutaminase 3 is expressed in basal cell carcinoma of the skin

Short title: **TG3 in skin cancer**

Artem Smirnov¹, Lucia Anemona¹, Manuela Montanaro¹, Alessandro Mauriello¹, Margherita Annicchiarico-Petruzzelli², Elena Campione³, Gerry Melino^{1,4}, and Eleonora Candi^{1,2}

¹ Department of Experimental Medicine, TOR, University of Rome "Tor Vergata", 00133 Rome, Italy

² Istituto Dermopatico dell'Immacolata-IRCCS, 00163 Rome, Italy

³ Department of Dermatology, University of Rome "Tor Vergata", 00133 Rome, Italy

⁴ MRC-Toxicology Unit, University of Cambridge, UK

Corresponding author: Eleonora Candi, email: candi@uniroma2.it, tel.: +390672596487

Words: 4550

Figures: 4

Tables: n/a

References: 43

Author

Artem Smirnov
Lucia Anemona
Manuela Montanaro
Alessandro Mauriello
Margherita Annicchiarico-Petruzzelli
Elena Campione
Gerry Melino
Eleonora Candi

Email

art.smirnow@gmail.com
anemona@uniroma2.it
manuelamontanaro1991@gmail.com
alessandro.mauriello@uniroma2.it
m.annicchiaricopetruzzelli@idi.it
campioneelena@hotmail.com
gm614@mrc-tox.cam.ac.uk
candi@uniroma2.it

ORCID

0000-0002-1575-8725
0000-0002-3711-2714
n/a
0000-0002-7351-5676
0000-0002-4717-4740
0000-0001-7447-6798
0000-0001-9428-5972
0000-0001-8332-4825

ABSTRACT

Background Transglutaminase 3 belongs to a family of Ca^{2+} -dependent enzymes which catalyse protein crosslinking. It is important for proper development of the skin and the hair shaft. Knock-out mice for *Tgm3* gene are more sensitive to UVB-induced photodamage due to aberrations in cornified envelope formation. Loss of TG3 was found in head and neck and oesophageal squamous cell carcinoma, yet, its expression in skin cancer has not been studied.

Objectives The aim of present study is to analyse the expression pattern of TG3 in skin cancer.

Materials & Methods We conducted immunohistochemical staining to detect TG3 expression using tissue microarray of different types of skin cancer as well as meta-analysis of public gene array data.

Results Our findings demonstrated that TG3 is normally expressed in spinous/granular layers of the epidermis, meanwhile is absent in melanocytes as well as melanoma samples. As expected, its expression was found lost in poorly differentiated squamous cell carcinoma of the skin. Surprisingly, we show that samples of basal cell carcinoma had a strong staining for TG3 both in cytoplasm and in the nucleus. Furthermore, at mRNA level the expression pattern of *TGM3* is crucially altered in BCC, but not other types of skin cancer.

Conclusion These findings open new questions regarding TG3 involvement in basal cell carcinoma tumorigenesis. Moreover, its expression pattern renders TG3 a potential specific marker for basal cell carcinoma diagnosis.

Keywords: basal cell carcinoma, keratinocytes, melanoma, skin cancer, squamous cell carcinoma, Transglutaminase 3.

Abbreviation *TGM3*, transglutaminase 3 gene; TG3 transglutaminase 3; *H&E*, haematoxylin and eosin staining; *TMA*, tissue micro-array; *BCC*, basal cell carcinoma; *SCC*, squamous cell carcinoma; *mel*, cutaneous melanoma; *H-score*, histological score of protein expression.

INTRODUCTION

The family of transglutaminases (TGs) is composed of Ca^{2+} -dependent enzymes whose main function is to catalyse protein crosslinking [1]. Transglutaminase 3 (TG3) is expressed in differentiated layer of the epidermis and hair follicle. During keratinocyte differentiation, similarly to the other TG members, TG1 and TG5, [2–4], proteolysis is required for TG3 activation [5]. It was demonstrated that Sp1 and ETS-transcription factors promote *TGM3* expression in squamous epithelia [6], moreover, two different transcription variants of *TGM3* were identified [7]. TG3 together with TG1 has as main protein substrates loricrin, involucrin, and the family of small-proline rich proteins (SPRs). The crosslinking between loricrin and SPRs makes part of cornification of the skin [3]. Even though *Tgm3*^{-/-} mice do not show any strong abnormalities in the skin formation, a more invasive percutaneous penetration of FITC in the KO mice was observed [8]. Recently, our laboratory demonstrated that the absence of Tg3 sensitizes the skin for damage induced by UVB irradiation [9]. Moreover, a mutation within *TGM3* gene was shown to be one of the causes of uncombable hair syndrome [10].

Multiple studies demonstrated the downregulation of TG3 both at RNA and protein levels in head and neck squamous cell carcinomas [11–17]. As a molecular mechanism of TG3 repression, was proposed the hypermethylation of CpG islands within *TGM3* promoter [18]. Furthermore, *TGM3* was shown as putative tumour-suppressor gene in the oesophageal cancer, in which its expression was found downregulated and associated with tumour proliferation and migration [19,20].

Skin cancer is the most common type of cancer. There are three different major types of skin cancer: basal cell carcinoma, squamous cell carcinoma, referred as non-melanoma skin cancer, and malignant melanoma. Basal cell carcinoma is the most common type of cancer in the world. It preferentially arises from stem cell of the hair follicle [21], meanwhile squamous cell carcinoma arises from epidermal keratinocytes and it can be more aggressive and give metastasis [22]. Melanoma arises from melanocytes, its incidence is minor respect to non-melanoma skin cancers, but it is the deadliest form of skin cancer [23]. Recently, a SNP has been found within *TGM3* gene and it is associated with higher risk of basal cell carcinoma incidence among the Icelanders [24]. However, the expression of TG3 in skin cancers has not been investigated yet.

Here, using tissue-micro array (TMA) approach combined with meta-analysis of skin cancer datasets, we investigated TG3 expression in different patients. We observed its down-regulation in melanoma and aggressive squamous cell carcinomas while we found TG3 high expressed in basal cell carcinoma, making TG3 suitable as biomarker for this type of skin cancer.

MATERIALS AND METHODS

Immunohistochemical staining

Skin cancer tissue microarray, containing 10 samples of normal skin, 10 samples of malignant melanoma, 39 samples of cutaneous squamous cell carcinoma, and 13 samples of cutaneous basal cell carcinoma, was purchased from US Biomax (Cat. No. SK801c, Rockville, MD, USA). Other samples of cBCC from this study were utilized with the approval (Protocol No. 130/18) of the institutional review board of University Hospital “Policlinico Tor Vergata” (Rome, Italy) and prior patient consent. The immunohistochemical staining for Ki67 and Ep-CAM were performed using anti-Ki67 antibody (Cat. No. 790-4286, Ventana, Oro Valley, AZ, USA) and anti-Ep-CAM antibody (Cat. No. 760-4383, Ventana) following manufacturer’s indications in the automatized BenchMark ULTRA slide staining system (Ventana). For TG3 staining, sections were dewaxed and rehydrated, incubated for blocking of endogenous peroxidases in 0.03% solution of hydrogen peroxide in

methanol, then the antigen retrieval was performed by boiling the sample in the 0.01 M citrate buffer pH 6.0 for 10 min in microwave. Slides were incubated with anti-TG3 antibody (1:300, Cat. No. C2D, Covalab, Villeurbanne, France) for 20 min at room temperature. The signal was detected using UltraTek HRP anti-polivalent DAB staining system (ScyTek, Logan, UT, USA), then the slides were counterstained with haematoxylin, dehydrated and mounted. Slides were scanned using 40x objective in the Ventana iCoreo scanner (Ventana). Haematoxylin/eosin staining images for TMA were downloaded from the manufacturer's web-site (www.biomax.us).

Immunofluorescence and confocal microscopy

Paraffin-embedded sections of BCC were dewaxed and rehydrated, then the antigen retrieval was performed by boiling the sample in the 0.01 M citrate buffer pH 6.0 for 10 min in microwave. To reduce tissue autofluorescence, samples were incubated for 45 min in 0.1 M sodium tetraborate solution followed by 1 h of blocking with 5% goat serum in PBS. Slides were incubated with anti-TG3 antibody (1:300) and anti-Loricrin (1:300, Poly19051, BioLegend, San Diego, CA, USA) overnight at 4°C. Then, sections were incubated for 1 h at room temperature with secondary anti-mouse and anti-rabbit 488- or 568-AlexaFluor conjugated antibodies (1:1000, Invitrogen, Carlsbad, CA, USA) together with 1 µg/mL DAPI (Sigma, St. Louis, MO, USA) for nuclear DNA staining. Sections were analysed with a confocal laser microscope (NIKON Eclipse Ti) using NIS-Elements AR Ver. 4.4 software (Nikon, Tokyo, Japan).

Histological scoring of the samples

Samples were scored in a blinded manner by a pathologist using a semi-quantitative method. Cases were analysed for staining intensity, which was scored as 0 (not detected), 1+ (weak), 2+ (intermediate), and 3+ (strong). For each case, the histological "H-score" (0-300) was calculated by multiplying the percentage of positive cells (0%-100%) by the intensity (0-3). Percentage of Ki67 positive samples was calculated as the number of Ki67 positive neoplastic cells per total number of neoplastic cells x 100%. Ten random fields were analysed for each sample.

Bioinformatic analysis

Normalized values of *TGM1*, *TGM3*, *TGM5*, *LOR*, and *IVL* expression in the skin cancer samples were obtained from NCBI GEO portal (accession number: GSE7553, [25]). The analysis of co-expression was performed using publicly available on-line platform "R2: Genomics Analysis and Visualization Platform" (r2.amc.nl). The Gene Ontology analysis was carried out using DAVID on-line platform (david.ncifcrf.gov).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 7.0 Software (San Diego, CA, USA). For the analysis of gene array data and TG3 protein level from the tissue microarray experiment, the significance level (*P*) was calculated using Welch's unequal variances *t*-test. Values of *P* < 0.05 were considered significant. Violin plots were generated in R using ggplot2 package.

RESULTS

TG3 is differently expressed in skin cancer

Previously published data revealed an important role of TG3 in the oesophageal and head and neck cancer [14,19], meanwhile its expression pattern in the skin cancer remains unveiled. To analyse the expression of TG3 at protein level, we used a tissue micro-array containing normal skin, cutaneous melanoma, squamous cell carcinoma (SCC), and basal cell carcinoma (BCC). Firstly, we performed the immunohistochemical staining of TG3 using 10 samples of normal human skin (**Fig. 1A**). We observed a homogeneous distribution of the staining among all specimens regardless the patients' age, sex, or biopsy anatomic site (**Supplementary Table 1**). In line with previous studies [26], all samples showed the localisation of TG3 in the upper spinous and granular layers, meanwhile basal and cornified layers, as well as dermis were found negative for TG3. As a marker of proliferation, we used Ki67. Then, we analysed the expression of TG3 in tumour samples. We found almost all samples of melanoma negative for TG3 staining (**Fig. 1B**, **Supplementary Table 1**). Then, we carried out the analysis of TG3 expression in the SCC samples (**Fig. 1C**, **Supplementary Table 1**). The majority of them (24/39) were negative for TG3. Several samples of low-grade and well-differentiated tumours showed positive staining only in the differentiated cells respect to the poorly-differentiated samples. Surprisingly, we found out a very heterogenous distribution of TG3 expression in the BCC samples (**Fig. 1D**, **Supplementary Table 1**). Meanwhile, several samples were negative (4/13) or weakly-stained (4/13) for TG3, almost the half showed a very strong staining (5/13). However, more precise analysis of the samples in blinded manner by three independent pathologists (University Hospital "Policlinico Tor Vergata", Rome, Italy) did not give certain conclusions on the diagnosis of TG3 negative samples. To resolve this problem, we performed the immunohistochemical staining for Ep-CAM, which was shown to be a specific marker for basal cell carcinoma of the skin [27]. Surprisingly, only some of them (4/13) were positive (H-score > 100) for this protein (**Supplementary Table 1**). Hence, we decided to perform additional staining for six BCC sections from the Pathology unit of "Policlinico Tor Vergata" Hospital (Rome, Italy). We found all samples strongly positive for both Ep-CAM and TG3 (**Fig. 2A**). Interestingly, in TG3-positive samples, its localisation was not only cytoplasmic but also nuclear. Further statistical analysis of TG3 expression in the skin cancer tissue micro-array and additional cases (**Fig. 2B-C**) revealed a strong decrease of TG3 level in melanoma ($P=5.3 \times 10^{-6}$) and SCC ($P=5.9 \times 10^{-6}$) samples respect to the normal epidermis. On the other hand, TG3 level in the BCC samples (only Ep-CAM positive samples from TMA and additional cases) was significantly increased respect to the normal epidermis ($FC=2.2$, $P=6.5 \times 10^{-5}$).

Differentiation-related profile of *TGM3* expression is lost in BCC, but not in SCC

For further confirmation of our observations, we analysed the expression of *TGM3* at mRNA level in normal skin and skin cancers from publicly available gene array (accession number: GSE7553, [25] **Fig. 3A**). We observed that *TGM3* expression is significantly decreased in melanoma samples ($P=0.026$), meanwhile there were no significant changes in *TGM3* expression in the SCC ($P=0.557$). In line with our results, we observed a significant 2-fold increase of *TGM3* level in the basal cell carcinoma samples ($P=0.011$). Hence, we decided to investigate whether the expression of other skin-related TGs (as *TGM1* and *TGM5*) or their common substrates (as *LOR* and *IVL*) was altered in BCC respect to the normal skin. Our results showed no significant changes in the expression of any of genes analysed ($P>0.15$), indicating that increased expression of TG3 in BCC is specific and

not linked to a de-regulation of the differentiation process (**Fig. 3B**). To confirm these data at tissue level, we performed a co-staining of a BCC sample for TG3 and its substrate loricrin. As highlighted in the **Fig. 3C**, TG3 and loricrin are partly co-expressed in the upper layers of the skin adjacent to the tumour, meanwhile the tumour regions are positive for TG3 and completely negative for loricrin. To support the observation that TG3 is uniquely overexpressed in BCC independently on other differentiation-related genes, we performed the correlation analysis of expression of *TGM1*, *TGM3*, *TGM5*, *LOR*, and *IVL* in normal skin and skin cancer using the data from the gene array (GSE7553). As expected, we saw a high correlation ($R > +0.75$) between the expression of all genes analysed in normal skin, melanoma, and SCC samples (**Fig. 4A**). Surprisingly, in BCC samples, we found this trend maintained only between *TGM1*, *TGM5*, *LOR*, and *IVL* ($R > +0.75$), but not *TGM3* ($R \approx 0$). The gene ontology analysis (**Fig. 4B**) revealed that top 300 genes co-expressed with TG's substrate *LOR* are strongly related to epidermal differentiation cluster in all types of skin cancer ($P: 10^{-4} \div 10^{-50}$). Interestingly, this trend can be noticed also for *TGM3* but only in melanoma and SCC samples ($P: 10^{-2} \div 10^{-50}$), meanwhile differentiation-related pattern of *TGM3* expression is completely lost in BCC samples (GO groups: “aromatic/nitrogen/lipid compound biosynthesis”, “water-soluble vitamin metabolism”, etc., $P > 10^{-3}$). These data indicate that, unlike other skin cancers, in BCC, despite the strong de-regulation of TG3 expression, other proteins related to keratinization are not altered.

DISCUSSION

Skin differentiation is a specialized form of cell death, unlike apoptosis, p53 and Bcl2 [28,29,38,30–37] and other classical pro-apoptotic effectors are not involved, while TG enzymes (TG1, TG3, and TG5) play a crucial role [2,4–6,39]. TG3 is a Ca^{2+} -dependent enzyme important for protein crosslinking. It is mainly expressed in squamous epithelia such as the epidermis as well as in the hair follicles. Its crosslinking activity contributes to the formation of the cornified cell envelope and the hair shaft [3]. Interestingly, the skin of *Tgm3*^{-/-} mice shows higher permeability and is more sensitized to photodamage induced by UVB-irradiation [8,9]. Moreover, loss of TG3 was described in head and neck and oesophageal squamous cell carcinoma [18,19], indicating its possible involvement in epithelial cancers. However, TG3 role in skin cancer remains unrevealed, thus we investigated its expression pattern in this pathology.

Firstly, we performed an immunohistochemical staining using tissue micro-array, containing different types of skin cancer as well as normal skin controls. We observed positive staining for TG3 in upper spinous and granular layers of normal epidermis, meanwhile basal layer cells as well as melanocytes were found negative. Indeed, melanoma samples maintained negative staining for TG3 as expected due to non-squamous origin of this type of skin cancer. Interestingly, most of squamous cell carcinoma samples were found negative for TG3 as well. The latter confirms previously published data [18,19]. To note, SCC that stained positively for TG3 were the well differentiated ones, around the keratin pearls. Conversely, we found a very strong signal for TG3 in basal cell carcinoma samples. Surprisingly, in these samples TG3 was detected both in the cytoplasm and in the nucleus. Since BCC carcinogenesis shares several common features with hair development, additional analysis of TG3 expression and enzymatic activity must be performed during the initial steps of hair follicle development. Moreover, abnormal nuclear localisation of TG3 in tumour cells indicates possible existence of additional activities of this enzyme which are uncommon for differentiated epidermal cells.

Further investigation of *TGM3* expression at mRNA level in skin cancer revealed a dramatic de-regulation of its differentiation-related pattern of expression only in BCC samples, but not in SCC or melanoma. Interestingly, the expression of other related genes as *TGM1*, *TGM5*, *LOR*, or *IVL*

remained strongly correlated with differentiation in all samples, hence de-regulation of *TGM3* expression cannot be explained by aberrations in differentiation programme, **but implies the existence of additional transcriptional activities which trigger *TGM3* expression exclusively in BCC tumorigenesis**. More accurate analysis reveals that the cells of the basal layer of normal skin, which give origin to BCC, are negative for TG3 in contrast to tumour cells. This observation indicates that during BCC initiation several pathways lead to the transcriptional activation of *TGM3* expression. BCC are characterised by the altered Hedgehog pathway and, as a consequence, abnormal activity of GLI transcription factors [40]. Of interest, several binding sites for GLI2 were identified within promoter region of *TGM3* [41,42]. Hence, a possible scenario of abnormal TG3 expression in BCC could be related to the transcriptional activation of *TGM3* by GLI2. However, the exact mechanisms for TG3 regulation remain completely elusive.

To note, TG3 expression pattern is similar to the expression of Ep-CAM, specifically found in this type of skin cancer. In fact, routinely, BCC diagnosis is also confirmed by positive Ep-CAM staining [43]. Ep-CAM is absent in normal skin, therefore a negative staining for Ep-CAM in the tissue could be also the result of an inappropriate fixation or processing of the samples which leads to uncertain conclusions regarding the diagnosis. Being expressed both in BCC cells and in granular layer of normal epidermis, TG3 staining of adjacent skin can serve as an internal positive control for the antibody and sample preparation, rendering TG3 potentially more accurate diagnostic marker for BCC respect to Ep-CAM.

Altogether, our findings demonstrate an abnormal overexpression of TG3 in basal cell carcinoma. Further research is necessary to support these observations with higher number of samples and to reveal molecular mechanisms of this phenomenon. The data presented indicate TG3 as a new potential specific marker for the diagnosis of cutaneous basal cell carcinoma.

ACKNOWLEDGEMENTS

We thank Caterina Marcelli, Dr. Manuel Scimeca, and Dr. Andrea Saggini (University Hospital “Policlinico Tor Vergata”, Rome, Italy) for technical assistance and discussion. This work has been mainly supported by IDI-IRCCS, RC to EC and partially supported by AIRC grant to EC (IG22206). The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

AS, LA, MM, and EC performed the research; AS, LA, AM, MAP, and GM analysed the data; EC designed the research and wrote the paper; and all the authors read the paper and made comments.

REFERENCES

1. Lorand L, Graham RM. Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nature Reviews Molecular Cell Biology* 2003; **4**: 140–56.
2. Candi E, Melino G, Lahm A, Ceci R, Rossi A, Kim IG et al. Transglutaminase 1 mutations in lamellar ichthyosis: Loss of activity due to failure of activation by proteolytic processing. *Journal of Biological Chemistry* 1998; **273**: 13693–702.
3. Candi E, Tarcsa E, Idler WW, Kartasova T, Marekov LN, Steinert PM. Transglutaminase cross-linking properties of the small proline-rich 1 family of cornified cell envelope proteins: Integration with loricrin. *Journal of Biological Chemistry* 1999; **274**: 7226–37.
4. Candi E, Oddi S, Terrinoni A, Paradisi A, Ranalli M, Finazzi-Agró A et al. Transglutaminase 5 Cross-links Loricrin, Involucrin, and Small Proline-rich Proteins in Vitro. *Journal of Biological Chemistry* 2001; **276**: 35014–

23.

5. Kim IG, Lee SC, Lee JH, Yang JM, Chung S II, Steinert PM. Structure and organization of the human transglutaminase 3 gene: Evolutionary relationship to the transglutaminase family. *Journal of Investigative Dermatology* 1994; **103**: 137–42.
6. Lee JH, Jang SI, Yang JM, Markova NG, Steinert PM. The proximal promoter of the human transglutaminase 3 gene: Stratified squamous epithelial-specific expression in cultured cells is mediated by binding of Sp1 and ets transcription factors to a proximal promoter element. *Journal of Biological Chemistry* 1996; **271**: 4561–8.
7. Zocchi L, Terrinoni A, Candi E, Ahvaz B, Bagetta G, Corasaniti M et al. Identification of transglutaminase 3 splicing isoforms. *Journal of Investigative Dermatology* 2007; **127**: 1791–4.
8. Bogнар P, Nemeth I, Mayer B, Haluszka D, Wikonkal N, Ostorhazi E et al. Reduced inflammatory threshold indicates skin barrier defect in transglutaminase 3 knockout mice. *Journal of Investigative Dermatology* 2014; **134**: 105–11.
9. Frezza V, Terrinoni A, Pitolli C, Mauriello A, Melino G, Candi E. Transglutaminase 3 Protects against Photodamage. *Journal of Investigative Dermatology* 2017; **137**: 1590–4.
10. Ü. Basmanav FB, Cau L, Tafazzoli A, Méchin MC, Wolf S, Romano MT et al. Mutations in Three Genes Encoding Proteins Involved in Hair Shaft Formation Cause Uncombable Hair Syndrome. *American Journal of Human Genetics* 2016; **99**: 1292–304.
11. To G. Tumor-specific genetic expression profile of metastatic oral squamous cell carcinoma. *Head Neck* 2016; **29**: 1–18.
12. Choi P, Jordan CD, Mendez E, Houck J, Yueh B, Farwell DG et al. Examination of oral cancer biomarkers by tissue microarray analysis. *Archives of Otolaryngology - Head and Neck Surgery* 2008; **134**: 539–46.
13. Lallemand B, Evrard A, Combescure C, Chapuis H, Chambon G, Raynal C et al. Clinical relevance of nine transcriptional molecular markers for the diagnosis of head and neck squamous cell carcinoma in tissue and saliva rinse. *BMC Cancer* 2009; **9**: 370.
14. Wu X, Cao W, Wang X, Zhang J, Lv Z, Qin X et al. TGM3, a candidate tumor suppressor gene, contributes to human head and neck cancer. *Molecular Cancer* 2013; **12**: 151.
15. Luo A, Kong J, Hu G, Liew CC, Xiong M, Wang X et al. Discovery of Ca²⁺-relevant and differentiation-associated genes downregulated in esophageal squamous cell carcinoma using cDNA microarray. *Oncogene* 2004; **23**: 1291–9.
16. Bundela S, Sharma A, Bisen PS. Potential therapeutic targets for oral cancer: ADM, TP53, EGFR, LYN, CTLA4, SKIL, CTGF, CD70. *PLoS ONE* 2014; **9**.
17. Nair J, Jain P, Chandola U, Palve V, Harsha Vardhan NR, Reddy RB et al. Gene and miRNA expression changes in squamous cell carcinoma of larynx and hypopharynx. *Genes & Cancer* 2015; **6**: 328–40.
18. Negishi A, Masuda M, Ono M, Honda K, Shitashige M, Satow R et al. Quantitative proteomics using formalin-fixed paraffin-embedded tissues of oral squamous cell carcinoma. *Cancer Science* 2009; **100**: 1605–11.
19. Uemura N, Nakanishi Y, Kato H, Saito S, Nagino M, Hirohashi S et al. Transglutaminase 3 as a prognostic biomarker in esophageal cancer revealed by proteomics. *International Journal of Cancer* 2009; **124**: 2106–15.
20. Li W, Zhang Z, Zhao W, Han N. Transglutaminase 3 protein modulates human esophageal cancer cell growth by targeting the NF-κB signaling pathway. *Oncology Reports* 2016; **36**: 1723–30.
21. Peterson SC, Eberl M, Vagnozzi AN, Belkadi A, Veniaminova NA, Verhaegen ME et al. Basal cell carcinoma preferentially arises from stem cells within hair follicle and mechanosensory niches. *Cell Stem Cell* 2015; **16**: 400–12.
22. Khavari PA. Modelling cancer in human skin tissue. *Nature Reviews Cancer* 2006; **6**: 270–80.
23. Owens B. Melanoma. *Nature* 2014; **515**: S109–S109.
24. Stacey SN, Sulem P, Gudbjartsson DF, Jonasdottir A, Thorleifsson G, Gudjonsson SA et al. Germline sequence variants in TGM3 and RGS22 confer risk of basal cell carcinoma. *Human Molecular Genetics* 2014; **23**: 3045–53.
25. Riker AI, Enkemann SA, Fodstad O, Liu S, Ren S, Morris C et al. The gene expression profiles of primary

and metastatic melanoma yields a transition point of tumor progression and metastasis. *BMC Medical Genomics* 2008; **1**: 13.

26. Hitomi K, Presland RB, Nakayama T, Fleckman P, Dale BA, Maki M. Analysis of epidermal-type transglutaminase (transglutaminase 3) in human stratified epithelia and cultured keratinocytes using monoclonal antibodies. *Journal of Dermatological Science* 2003; **32**: 95–103.

27. Dasgeb B, Mohammadi TM, Mehregan DR. Use of Ber-EP4 and Epithelial Specific Antigen to Differentiate Clinical Simulators of Basal Cell Carcinoma. *Biomarkers in Cancer* 2013; **5**: BIC.S11856.

28. Aubrey BJ, Kelly GL, Janic A, Herold MJ, Strasser A. How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression? *Cell Death and Differentiation* 2018; **25**: 104–13.

29. Baugh EH, Ke H, Levine AJ, Bonneau RA, Chan CS. Why are there hotspot mutations in the TP53 gene in human cancers? *Cell Death and Differentiation* 2018; **25**: 154–60.

30. Charni M, Aloni-Grinstein R, Molchadsky A, Rotter V. P53 on the crossroad between regeneration and cancer. *Cell Death and Differentiation* 2017; **24**: 8–14.

31. Engeland K. Cell cycle arrest through indirect transcriptional repression by p53: I have a DREAM. *Cell Death and Differentiation* 2018; **25**: 114–32.

32. Sullivan KD, Galbraith MD, Andrysk Z, Espinosa JM. Mechanisms of transcriptional regulation by p53. *Cell Death and Differentiation* 2018; **25**: 133–43.

33. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P et al. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death and Differentiation* 2018; **25**: 486–541.

34. Kim MP, Lozano G. Mutant p53 partners in crime. *Cell Death and Differentiation* 2018; **25**: 161–8.

35. Adams JM, Cory S. The BCL-2 arbiters of apoptosis and their growing role as cancer targets. *Cell Death and Differentiation* 2018; **25**: 27–36.

36. Kalkavan H, Green DR. MOMP, cell suicide as a BCL-2 family business. *Cell Death and Differentiation* 2018; **25**: 46–55.

37. Pihán P, Carreras-Sureda A, Hetz C. BCL-2 family: Integrating stress responses at the ER to control cell demise. *Cell Death and Differentiation* 2017; **24**: 1478–87.

38. Strasser A, Vaux DL. Viewing BCL2 and cell death control from an evolutionary perspective. *Cell Death and Differentiation*, 2018.

39. Ahvazi B, Boeshans KM, Idler W, Baxa U, Steinert PM. Roles of calcium ions in the activation and activity of the transglutaminase 3 enzyme. *Journal of Biological Chemistry* 2003; **278**: 23834–41.

40. Ikram MS, Neill GW, Regl G, Eichberger T, Frischauf AM, Aberger F et al. GLI2 is expressed in normal human epidermis and BCC and induces GLI1 expression by binding to its promoter. *Journal of Investigative Dermatology* 2004; **122**: 1503–9.

41. Winklmayr M, Schmid C, Laner-Plamberger S, Kaser A, Aberger F, Eichberger T et al. Non-consensus GLI binding sites in Hedgehog target gene regulation. *BMC Molecular Biology* 2010; **11**.

42. Laner-Plamberger S, Kaser A, Paulischta M, Hauser-Kronberger C, Eichberger T, Frischauf AM. Cooperation between GLI and JUN enhances transcription of JUN and selected GLI target genes. *Oncogene* 2009; **28**: 1639–51.

43. Sunjaya AP, Sunjaya AF, Tan ST. The Use of BEREPA Immunohistochemistry Staining for Detection of Basal Cell Carcinoma. *Journal of Skin Cancer*, 2017.

FIGURE LEGENDS

Figure 1 TG3 is differently expressed in skin cancer

(A) H&E staining and immunohistochemical analysis of Ki67 and TG3 expression in the normal skin ($n=10$) from TMA. **(B)** H&E staining and immunohistochemical analysis of Ki67 and TG3 expression in the cutaneous melanoma samples ($n=10$) from TMA. **(C)** H&E staining and immunohistochemical analysis of Ki67 and TG3 expression in the cutaneous squamous cell carcinoma ($n=39$) from TMA. Two representative cases of poorly- and well-differentiated tumours are shown. **(D)** H&E staining and immunohistochemical analysis of Ki67, TG3, and Ep-CAM expression in the cutaneous basal cell carcinoma ($n=13$) from TMA. Two representative cases with low and high TG3 expression are shown.

Figure 2 TG3 is highly expressed in Ep-CAM+ basal cell carcinoma

(A) H&E staining and immunohistochemical analysis of Ki67, TG3, and Ep-CAM expression in the cutaneous basal cell carcinoma ($n=6$). **(B)** Table showing the median H-score for TG3 and fold change enrichment of TG3 expression in tumour samples respect to normal skin. **(C)** Violin plot showing TG3 H-score distribution in the samples of normal skin and skin cancer from **B**.

Figure 3 *TGM3* is uniquely overexpressed in BCC respect to other TGs and its substrates

(A) Violin plot showing relative mRNA expression level of *TGM3* in normal and skin cancer samples from GSE7553. **(B)** Violin plot showing relative mRNA expression levels of *TGM1*, *TGM5*, *LOR*, and *IVL* in normal and BCC samples from GSE7553. **(C)** Immunofluorescence analysis of TG3 and LOR expression in a case of BCC. Yellow dashed line separates epidermis and dermis, white dashed lines indicate the tumour regions.

Figure 4 Differentiation-correlated profile of *TGM3* expression is lost in BCC, but not in SCC

(A) Heatmaps showing the correlation of expression between *TGM1*, *TGM3*, *TGM5*, *LOR*, and *IVL* in normal skin and different types of skin cancer from gene array GSE7553. **(B)** GO terms analysis of the top 300 genes co-expressed with either *LOR* or *TGM3* in melanoma, BCC, and SCC from gene array GSE7553. Pearson's correlation $R > +0.50$.

Transglutaminase 3 is expressed in basal cell carcinoma of the skin

Short title: **TG3 in skin cancer**

Artem Smirnov¹, Lucia Anemona¹, Manuela Montanaro¹, Alessandro Mauriello¹, Margherita Annicchiarico-Petruzzelli², Elena Campione³, Gerry Melino^{1,4}, and Eleonora Candi^{1,2}

¹ Department of Experimental Medicine, TOR, University of Rome "Tor Vergata", 00133 Rome, Italy

² Istituto Dermopatico dell'Immacolata-IRCCS, 00163 Rome, Italy

³ Department of Dermatology, University of Rome "Tor Vergata", 00133 Rome, Italy

⁴ MRC-Toxicology Unit, University of Cambridge, UK

Corresponding author: Eleonora Candi, email: candi@uniroma2.it, tel.: +390672596487

Words: 4550

Figures: 4

Tables: n/a

References: 43

Author

Artem Smirnov
Lucia Anemona
Manuela Montanaro
Alessandro Mauriello
Margherita Annicchiarico-Petruzzelli
Elena Campione
Gerry Melino
Eleonora Candi

Email

art.smirnow@gmail.com
anemona@uniroma2.it
manuelamontanaro1991@gmail.com
alessandro.mauriello@uniroma2.it
m.annicchiaricopetruzzelli@idi.it
campioneelena@hotmail.com
gm614@mrc-tox.cam.ac.uk
candi@uniroma2.it

ORCID

0000-0002-1575-8725
0000-0002-3711-2714
n/a
0000-0002-7351-5676
0000-0002-4717-4740
0000-0001-7447-6798
0000-0001-9428-5972
0000-0001-8332-4825

ABSTRACT

Background Transglutaminase 3 belongs to a family of Ca^{2+} -dependent enzymes which catalyse protein crosslinking. It is important for proper development of the skin and the hair shaft. Knock-out mice for *Tgm3* gene are more sensitive to UVB-induced photodamage due to aberrations in cornified envelope formation. Loss of TG3 was found in head and neck and oesophageal squamous cell carcinoma, yet, its expression in skin cancer has not been studied.

Objectives The aim of present study is to analyse the expression pattern of TG3 in skin cancer.

Materials & Methods We conducted immunohistochemical staining to detect TG3 expression using tissue microarray of different types of skin cancer as well as meta-analysis of public gene array data.

Results Our findings demonstrated that TG3 is normally expressed in spinous/granular layers of the epidermis, meanwhile is absent in melanocytes as well as melanoma samples. As expected, its expression was found lost in poorly differentiated squamous cell carcinoma of the skin. Surprisingly, we show that samples of basal cell carcinoma had a strong staining for TG3 both in cytoplasm and in the nucleus. Furthermore, at mRNA level the expression pattern of *TGM3* is crucially altered in BCC, but not other types of skin cancer.

Conclusion These findings open new questions regarding TG3 involvement in basal cell carcinoma tumorigenesis. Moreover, its expression pattern renders TG3 a potential specific marker for basal cell carcinoma diagnosis.

Keywords: basal cell carcinoma, keratinocytes, melanoma, skin cancer, squamous cell carcinoma, Transglutaminase 3.

Abbreviation *TGM3*, transglutaminase 3 gene; TG3 transglutaminase 3; *H&E*, haematoxylin and eosin staining; *TMA*, tissue micro-array; *BCC*, basal cell carcinoma; *SCC*, squamous cell carcinoma; *mel*, cutaneous melanoma; *H-score*, histological score of protein expression.

INTRODUCTION

The family of transglutaminases (TGs) is composed of Ca^{2+} -dependent enzymes whose main function is to catalyse protein crosslinking [1]. Transglutaminase 3 (TG3) is expressed in differentiated layer of the epidermis and hair follicle. During keratinocyte differentiation, similarly to the other TG members, TG1 and TG5, [2–4], proteolysis is required for TG3 activation [5]. It was demonstrated that Sp1 and ETS-transcription factors promote *TGM3* expression in squamous epithelia [6], moreover, two different transcription variants of *TGM3* were identified [7]. TG3 together with TG1 has as main protein substrates loricrin, involucrin, and the family of small-proline rich proteins (SPRs). The crosslinking between loricrin and SPRs makes part of cornification of the skin [3]. Even though *Tgm3*^{-/-} mice do not show any strong abnormalities in the skin formation, a more invasive percutaneous penetration of FITC in the KO mice was observed [8]. Recently, our laboratory demonstrated that the absence of Tg3 sensitizes the skin for damage induced by UVB irradiation [9]. Moreover, a mutation within *TGM3* gene was shown to be one of the causes of uncombable hair syndrome [10].

Multiple studies demonstrated the downregulation of TG3 both at RNA and protein levels in head and neck squamous cell carcinomas [11–17]. As a molecular mechanism of TG3 repression, was proposed the hypermethylation of CpG islands within *TGM3* promoter [18]. Furthermore, *TGM3* was shown as putative tumour-suppressor gene in the oesophageal cancer, in which its expression was found downregulated and associated with tumour proliferation and migration [19,20].

Skin cancer is the most common type of cancer. There are three different major types of skin cancer: basal cell carcinoma, squamous cell carcinoma, referred as non-melanoma skin cancer, and malignant melanoma. Basal cell carcinoma is the most common type of cancer in the world. It preferentially arises from stem cell of the hair follicle [21], meanwhile squamous cell carcinoma arises from epidermal keratinocytes and it can be more aggressive and give metastasis [22]. Melanoma arises from melanocytes, its incidence is minor respect to non-melanoma skin cancers, but it is the deadliest form of skin cancer [23]. Recently, a SNP has been found within *TGM3* gene and it is associated with higher risk of basal cell carcinoma incidence among the Icelanders [24]. However, the expression of TG3 in skin cancers has not been investigated yet.

Here, using tissue-micro array (TMA) approach combined with meta-analysis of skin cancer datasets, we investigated TG3 expression in different patients. We observed its down-regulation in melanoma and aggressive squamous cell carcinomas while we found TG3 high expressed in basal cell carcinoma, making TG3 suitable as biomarker for this type of skin cancer.

MATERIALS AND METHODS

Immunohistochemical staining

Skin cancer tissue microarray, containing 10 samples of normal skin, 10 samples of malignant melanoma, 39 samples of cutaneous squamous cell carcinoma, and 13 samples of cutaneous basal cell carcinoma, was purchased from US Biomax (Cat. No. SK801c, Rockville, MD, USA). Other samples of cBCC from this study were utilized with the approval (Protocol No. 130/18) of the institutional review board of University Hospital “Policlinico Tor Vergata” (Rome, Italy) and prior patient consent. The immunohistochemical staining for Ki67 and Ep-CAM were performed using anti-Ki67 antibody (Cat. No. 790-4286, Ventana, Oro Valley, AZ, USA) and anti-Ep-CAM antibody (Cat. No. 760-4383, Ventana) following manufacturer’s indications in the automatized BenchMark ULTRA slide staining system (Ventana). For TG3 staining, sections were dewaxed and rehydrated, incubated for blocking of endogenous peroxidases in 0.03% solution of hydrogen peroxide in

methanol, then the antigen retrieval was performed by boiling the sample in the 0.01 M citrate buffer pH 6.0 for 10 min in microwave. Slides were incubated with anti-TG3 antibody (1:300, Cat. No. C2D, Covalab, Villeurbanne, France) for 20 min at room temperature. The signal was detected using UltraTek HRP anti-polivalent DAB staining system (ScyTek, Logan, UT, USA), then the slides were counterstained with haematoxylin, dehydrated and mounted. Slides were scanned using 40x objective in the Ventana iCoreo scanner (Ventana). Haematoxylin/eosin staining images for TMA were downloaded from the manufacturer's web-site (www.biomax.us).

Immunofluorescence and confocal microscopy

Paraffin-embedded sections of BCC were dewaxed and rehydrated, then the antigen retrieval was performed by boiling the sample in the 0.01 M citrate buffer pH 6.0 for 10 min in microwave. To reduce tissue autofluorescence, samples were incubated for 45 min in 0.1 M sodium tetrahydroborate solution followed by 1 h of blocking with 5% goat serum in PBS. Slides were incubated with anti-TG3 antibody (1:300) and anti-Loricrin (1:300, Poly19051, BioLegend, San Diego, CA, USA) overnight at 4°C. Then, sections were incubated for 1 h at room temperature with secondary anti-mouse and anti-rabbit 488- or 568-AlexaFluor conjugated antibodies (1:1000, Invitrogen, Carlsbad, CA, USA) together with 1 µg/mL DAPI (Sigma, St. Louis, MO, USA) for nuclear DNA staining. Sections were analysed with a confocal laser microscope (NIKON Eclipse Ti) using NIS-Elements AR Ver. 4.4 software (Nikon, Tokyo, Japan).

Histological scoring of the samples

Samples were scored in a blinded manner by a pathologist using a semi-quantitative method. Cases were analysed for staining intensity, which was scored as 0 (not detected), 1+ (weak), 2+ (intermediate), and 3+ (strong). For each case, the histological "H-score" (0-300) was calculated by multiplying the percentage of positive cells (0%-100%) by the intensity (0-3). Percentage of Ki67 positive samples was calculated as the number of Ki67 positive neoplastic cells per total number of neoplastic cells x 100%. Ten random fields were analysed for each sample.

Bioinformatic analysis

Normalized values of *TGM1*, *TGM3*, *TGM5*, *LOR*, and *IVL* expression in the skin cancer samples were obtained from NCBI GEO portal (accession number: GSE7553, [25]). The analysis of co-expression was performed using publicly available on-line platform "R2: Genomics Analysis and Visualization Platform" (r2.amc.nl). The Gene Ontology analysis was carried out using DAVID on-line platform (david.ncifcrf.gov).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 7.0 Software (San Diego, CA, USA). For the analysis of gene array data and TG3 protein level from the tissue microarray experiment, the significance level (*P*) was calculated using Welch's unequal variances *t*-test. Values of *P* < 0.05 were considered significant. Violin plots were generated in R using ggplot2 package.

RESULTS

TG3 is differently expressed in skin cancer

Previously published data revealed an important role of TG3 in the oesophageal and head and neck cancer [14,19], meanwhile its expression pattern in the skin cancer remains unveiled. To analyse the expression of TG3 at protein level, we used a tissue micro-array containing normal skin, cutaneous melanoma, squamous cell carcinoma (SCC), and basal cell carcinoma (BCC). Firstly, we performed the immunohistochemical staining of TG3 using 10 samples of normal human skin (**Fig. 1A**). We observed a homogeneous distribution of the staining among all specimens regardless the patients' age, sex, or biopsy anatomic site (**Supplementary Table 1**). In line with previous studies [26], all samples showed the localisation of TG3 in the upper spinous and granular layers, meanwhile basal and cornified layers, as well as dermis were found negative for TG3. As a marker of proliferation, we used Ki67. Then, we analysed the expression of TG3 in tumour samples. We found almost all samples of melanoma negative for TG3 staining (**Fig. 1B**, **Supplementary Table 1**). Then, we carried out the analysis of TG3 expression in the SCC samples (**Fig. 1C**, **Supplementary Table 1**). The majority of them (24/39) were negative for TG3. Several samples of low-grade and well-differentiated tumours showed positive staining only in the differentiated cells respect to the poorly-differentiated samples. Surprisingly, we found out a very heterogenous distribution of TG3 expression in the BCC samples (**Fig. 1D**, **Supplementary Table 1**). Meanwhile, several samples were negative (4/13) or weakly-stained (4/13) for TG3, almost the half showed a very strong staining (5/13). However, more precise analysis of the samples in blinded manner by three independent pathologists (University Hospital "Policlinico Tor Vergata", Rome, Italy) did not give certain conclusions on the diagnosis of TG3 negative samples. To resolve this problem, we performed the immunohistochemical staining for Ep-CAM, which was shown to be a specific marker for basal cell carcinoma of the skin [27]. Surprisingly, only some of them (4/13) were positive (H-score > 100) for this protein (**Supplementary Table 1**). Hence, we decided to perform additional staining for six BCC sections from the Pathology unit of "Policlinico Tor Vergata" Hospital (Rome, Italy). We found all samples strongly positive for both Ep-CAM and TG3 (**Fig. 2A**). Interestingly, in TG3-positive samples, its localisation was not only cytoplasmic but also nuclear. Further statistical analysis of TG3 expression in the skin cancer tissue micro-array and additional cases (**Fig. 2B-C**) revealed a strong decrease of TG3 level in melanoma ($P=5.3 \times 10^{-6}$) and SCC ($P=5.9 \times 10^{-6}$) samples respect to the normal epidermis. On the other hand, TG3 level in the BCC samples (only Ep-CAM positive samples from TMA and additional cases) was significantly increased respect to the normal epidermis ($FC=2.2$, $P=6.5 \times 10^{-5}$).

Differentiation-related profile of *TGM3* expression is lost in BCC, but not in SCC

For further confirmation of our observations, we analysed the expression of *TGM3* at mRNA level in normal skin and skin cancers from publicly available gene array (accession number: GSE7553, [25] **Fig. 3A**). We observed that *TGM3* expression is significantly decreased in melanoma samples ($P=0.026$), meanwhile there were no significant changes in *TGM3* expression in the SCC ($P=0.557$). In line with our results, we observed a significant 2-fold increase of *TGM3* level in the basal cell carcinoma samples ($P=0.011$). Hence, we decided to investigate whether the expression of other skin-related TGs (as *TGM1* and *TGM5*) or their common substrates (as *LOR* and *IVL*) was altered in BCC respect to the normal skin. Our results showed no significant changes in the expression of any of genes analysed ($P>0.15$), indicating that increased expression of TG3 in BCC is specific and

not linked to a de-regulation of the differentiation process (Fig. 3B). To confirm these data at tissue level, we performed a co-staining of a BCC sample for TG3 and its substrate loricrin. As highlighted in the Fig. 3C, TG3 and loricrin are partly co-expressed in the upper layers of the skin adjacent to the tumour, meanwhile the tumour regions are positive for TG3 and completely negative for loricrin. To support the observation that TG3 is uniquely overexpressed in BCC independently on other differentiation-related genes, we performed the correlation analysis of expression of *TGM1*, *TGM3*, *TGM5*, *LOR*, and *IVL* in normal skin and skin cancer using the data from the gene array (GSE7553). As expected, we saw a high correlation ($R > +0.75$) between the expression of all genes analysed in normal skin, melanoma, and SCC samples (Fig. 4A). Surprisingly, in BCC samples, we found this trend maintained only between *TGM1*, *TGM5*, *LOR*, and *IVL* ($R > +0.75$), but not *TGM3* ($R \approx 0$). The gene ontology analysis (Fig. 4B) revealed that top 300 genes co-expressed with TG's substrate *LOR* are strongly related to epidermal differentiation cluster in all types of skin cancer ($P: 10^{-4} \div 10^{-50}$). Interestingly, this trend can be noticed also for *TGM3* but only in melanoma and SCC samples ($P: 10^{-2} \div 10^{-50}$), meanwhile differentiation-related pattern of *TGM3* expression is completely lost in BCC samples (GO groups: “aromatic/nitrogen/lipid compound biosynthesis”, “water-soluble vitamin metabolism”, etc., $P > 10^{-3}$). These data indicate that, unlike other skin cancers, in BCC, despite the strong de-regulation of TG3 expression, other proteins related to keratinization are not altered.

DISCUSSION

Skin differentiation is a specialized form of cell death, unlike apoptosis, p53 and Bcl2 [28,29,38,30–37] and other classical pro-apoptotic effectors are not involved, while TG enzymes (TG1, TG3, and TG5) play a crucial role [2,4–6,39]. TG3 is a Ca^{2+} -dependent enzyme important for protein crosslinking. It is mainly expressed in squamous epithelia such as the epidermis as well as in the hair follicles. Its crosslinking activity contributes to the formation of the cornified cell envelope and the hair shaft [3]. Interestingly, the skin of *Tgm3*^{-/-} mice shows higher permeability and is more sensitized to photodamage induced by UVB-irradiation [8,9]. Moreover, loss of TG3 was described in head and neck and oesophageal squamous cell carcinoma [18,19], indicating its possible involvement in epithelial cancers. However, TG3 role in skin cancer remains unrevealed, thus we investigated its expression pattern in this pathology.

Firstly, we performed an immunohistochemical staining using tissue micro-array, containing different types of skin cancer as well as normal skin controls. We observed positive staining for TG3 in upper spinous and granular layers of normal epidermis, meanwhile basal layer cells as well as melanocytes were found negative. Indeed, melanoma samples maintained negative staining for TG3 as expected due to non-squamous origin of this type of skin cancer. Interestingly, most of squamous cell carcinoma samples were found negative for TG3 as well. The latter confirms previously published data [18,19]. To note, SCC that stained positively for TG3 were the well differentiated ones, around the keratin pearls. Conversely, we found a very strong signal for TG3 in basal cell carcinoma samples. Surprisingly, in these samples TG3 was detected both in the cytoplasm and in the nucleus. Since BCC carcinogenesis shares several common features with hair development, additional analysis of TG3 expression and enzymatic activity must be performed during the initial steps of hair follicle development. Moreover, abnormal nuclear localisation of TG3 in tumour cells indicates possible existence of additional activities of this enzyme which are uncommon for differentiated epidermal cells.

Further investigation of *TGM3* expression at mRNA level in skin cancer revealed a dramatic de-regulation of its differentiation-related pattern of expression only in BCC samples, but not in SCC or melanoma. Interestingly, the expression of other related genes as *TGM1*, *TGM5*, *LOR*, or *IVL*

remained strongly correlated with differentiation in all samples, hence de-regulation of *TGM3* expression cannot be explained by aberrations in differentiation programme, **but implies the existence of additional transcriptional activities which trigger *TGM3* expression exclusively in BCC tumorigenesis**. More accurate analysis reveals that the cells of the basal layer of normal skin, which give origin to BCC, are negative for TG3 in contrast to tumour cells. This observation indicates that during BCC initiation several pathways lead to the transcriptional activation of *TGM3* expression. BCC are characterised by the altered Hedgehog pathway and, as a consequence, abnormal activity of GLI transcription factors [40]. Of interest, several binding sites for GLI2 were identified within promoter region of *TGM3* [41,42]. Hence, a possible scenario of abnormal TG3 expression in BCC could be related to the transcriptional activation of *TGM3* by GLI2. However, the exact mechanisms for TG3 regulation remain completely elusive.

To note, TG3 expression pattern is similar to the expression of Ep-CAM, specifically found in this type of skin cancer. In fact, routinely, BCC diagnosis is also confirmed by positive Ep-CAM staining [43]. Ep-CAM is absent in normal skin, therefore a negative staining for Ep-CAM in the tissue could be also the result of an inappropriate fixation or processing of the samples which leads to uncertain conclusions regarding the diagnosis. Being expressed both in BCC cells and in granular layer of normal epidermis, TG3 staining of adjacent skin can serve as an internal positive control for the antibody and sample preparation, rendering TG3 potentially more accurate diagnostic marker for BCC respect to Ep-CAM.

Altogether, our findings demonstrate an abnormal overexpression of TG3 in basal cell carcinoma. Further research is necessary to support these observations with higher number of samples and to reveal molecular mechanisms of this phenomenon. The data presented indicate TG3 as a new potential specific marker for the diagnosis of cutaneous basal cell carcinoma.

ACKNOWLEDGEMENTS

We thank Caterina Marcelli, Dr. Manuel Scimeca, and Dr. Andrea Saggini (University Hospital “Policlinico Tor Vergata”, Rome, Italy) for technical assistance and discussion. This work has been mainly supported by IDI-IRCCS, RC to EC and partially supported by AIRC grant to EC (IG22206). The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

AS, LA, MM, and EC performed the research; AS, LA, AM, MAP, and GM analysed the data; EC designed the research and wrote the paper; and all the authors read the paper and made comments.

REFERENCES

1. Lorand L, Graham RM. Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nature Reviews Molecular Cell Biology* 2003; **4**: 140–56.
2. Candi E, Melino G, Lahm A, Ceci R, Rossi A, Kim IG et al. Transglutaminase 1 mutations in lamellar ichthyosis: Loss of activity due to failure of activation by proteolytic processing. *Journal of Biological Chemistry* 1998; **273**: 13693–702.
3. Candi E, Tarcsa E, Idler WW, Kartasova T, Marekov LN, Steinert PM. Transglutaminase cross-linking properties of the small proline-rich 1 family of cornified cell envelope proteins: Integration with loricrin. *Journal of Biological Chemistry* 1999; **274**: 7226–37.
4. Candi E, Oddi S, Terrinoni A, Paradisi A, Ranalli M, Finazzi-Agró A et al. Transglutaminase 5 Cross-links Loricrin, Involucrin, and Small Proline-rich Proteins in Vitro. *Journal of Biological Chemistry* 2001; **276**: 35014–

23.

5. Kim IG, Lee SC, Lee JH, Yang JM, Chung S II, Steinert PM. Structure and organization of the human transglutaminase 3 gene: Evolutionary relationship to the transglutaminase family. *Journal of Investigative Dermatology* 1994; **103**: 137–42.
6. Lee JH, Jang SI, Yang JM, Markova NG, Steinert PM. The proximal promoter of the human transglutaminase 3 gene: Stratified squamous epithelial-specific expression in cultured cells is mediated by binding of Sp1 and ets transcription factors to a proximal promoter element. *Journal of Biological Chemistry* 1996; **271**: 4561–8.
7. Zocchi L, Terrinoni A, Candi E, Ahvaz B, Bagetta G, Corasaniti M et al. Identification of transglutaminase 3 splicing isoforms. *Journal of Investigative Dermatology* 2007; **127**: 1791–4.
8. Bogнар P, Nemeth I, Mayer B, Haluszka D, Wikonkal N, Ostorhazi E et al. Reduced inflammatory threshold indicates skin barrier defect in transglutaminase 3 knockout mice. *Journal of Investigative Dermatology* 2014; **134**: 105–11.
9. Frezza V, Terrinoni A, Pitolli C, Mauriello A, Melino G, Candi E. Transglutaminase 3 Protects against Photodamage. *Journal of Investigative Dermatology* 2017; **137**: 1590–4.
10. Ü. Basmanav FB, Cau L, Tafazzoli A, Méchin MC, Wolf S, Romano MT et al. Mutations in Three Genes Encoding Proteins Involved in Hair Shaft Formation Cause Uncombable Hair Syndrome. *American Journal of Human Genetics* 2016; **99**: 1292–304.
11. To G. Tumor-specific genetic expression profile of metastatic oral squamous cell carcinoma. *Head Neck* 2016; **29**: 1–18.
12. Choi P, Jordan CD, Mendez E, Houck J, Yueh B, Farwell DG et al. Examination of oral cancer biomarkers by tissue microarray analysis. *Archives of Otolaryngology - Head and Neck Surgery* 2008; **134**: 539–46.
13. Lallemand B, Evrard A, Combescure C, Chapuis H, Chambon G, Raynal C et al. Clinical relevance of nine transcriptional molecular markers for the diagnosis of head and neck squamous cell carcinoma in tissue and saliva rinse. *BMC Cancer* 2009; **9**: 370.
14. Wu X, Cao W, Wang X, Zhang J, Lv Z, Qin X et al. TGM3, a candidate tumor suppressor gene, contributes to human head and neck cancer. *Molecular Cancer* 2013; **12**: 151.
15. Luo A, Kong J, Hu G, Liew CC, Xiong M, Wang X et al. Discovery of Ca²⁺-relevant and differentiation-associated genes downregulated in esophageal squamous cell carcinoma using cDNA microarray. *Oncogene* 2004; **23**: 1291–9.
16. Bundela S, Sharma A, Bisen PS. Potential therapeutic targets for oral cancer: ADM, TP53, EGFR, LYN, CTLA4, SKIL, CTGF, CD70. *PLoS ONE* 2014; **9**.
17. Nair J, Jain P, Chandola U, Palve V, Harsha Vardhan NR, Reddy RB et al. Gene and miRNA expression changes in squamous cell carcinoma of larynx and hypopharynx. *Genes & Cancer* 2015; **6**: 328–40.
18. Negishi A, Masuda M, Ono M, Honda K, Shitashige M, Satow R et al. Quantitative proteomics using formalin-fixed paraffin-embedded tissues of oral squamous cell carcinoma. *Cancer Science* 2009; **100**: 1605–11.
19. Uemura N, Nakanishi Y, Kato H, Saito S, Nagino M, Hirohashi S et al. Transglutaminase 3 as a prognostic biomarker in esophageal cancer revealed by proteomics. *International Journal of Cancer* 2009; **124**: 2106–15.
20. Li W, Zhang Z, Zhao W, Han N. Transglutaminase 3 protein modulates human esophageal cancer cell growth by targeting the NF-κB signaling pathway. *Oncology Reports* 2016; **36**: 1723–30.
21. Peterson SC, Eberl M, Vagnozzi AN, Belkadi A, Veniaminova NA, Verhaegen ME et al. Basal cell carcinoma preferentially arises from stem cells within hair follicle and mechanosensory niches. *Cell Stem Cell* 2015; **16**: 400–12.
22. Khavari PA. Modelling cancer in human skin tissue. *Nature Reviews Cancer* 2006; **6**: 270–80.
23. Owens B. Melanoma. *Nature* 2014; **515**: S109–S109.
24. Stacey SN, Sulem P, Gudbjartsson DF, Jonasdottir A, Thorleifsson G, Gudjonsson SA et al. Germline sequence variants in TGM3 and RGS22 confer risk of basal cell carcinoma. *Human Molecular Genetics* 2014; **23**: 3045–53.
25. Riker AI, Enkemann SA, Fodstad O, Liu S, Ren S, Morris C et al. The gene expression profiles of primary

and metastatic melanoma yields a transition point of tumor progression and metastasis. *BMC Medical Genomics* 2008; **1**: 13.

26. Hitomi K, Presland RB, Nakayama T, Fleckman P, Dale BA, Maki M. Analysis of epidermal-type transglutaminase (transglutaminase 3) in human stratified epithelia and cultured keratinocytes using monoclonal antibodies. *Journal of Dermatological Science* 2003; **32**: 95–103.

27. Dasgeb B, Mohammadi TM, Mehregan DR. Use of Ber-EP4 and Epithelial Specific Antigen to Differentiate Clinical Simulators of Basal Cell Carcinoma. *Biomarkers in Cancer* 2013; **5**: BIC.S11856.

28. Aubrey BJ, Kelly GL, Janic A, Herold MJ, Strasser A. How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression? *Cell Death and Differentiation* 2018; **25**: 104–13.

29. Baugh EH, Ke H, Levine AJ, Bonneau RA, Chan CS. Why are there hotspot mutations in the TP53 gene in human cancers? *Cell Death and Differentiation* 2018; **25**: 154–60.

30. Charni M, Aloni-Grinstein R, Molchadsky A, Rotter V. P53 on the crossroad between regeneration and cancer. *Cell Death and Differentiation* 2017; **24**: 8–14.

31. Engeland K. Cell cycle arrest through indirect transcriptional repression by p53: I have a DREAM. *Cell Death and Differentiation* 2018; **25**: 114–32.

32. Sullivan KD, Galbraith MD, Andrysk Z, Espinosa JM. Mechanisms of transcriptional regulation by p53. *Cell Death and Differentiation* 2018; **25**: 133–43.

33. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P et al. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death and Differentiation* 2018; **25**: 486–541.

34. Kim MP, Lozano G. Mutant p53 partners in crime. *Cell Death and Differentiation* 2018; **25**: 161–8.

35. Adams JM, Cory S. The BCL-2 arbiters of apoptosis and their growing role as cancer targets. *Cell Death and Differentiation* 2018; **25**: 27–36.

36. Kalkavan H, Green DR. MOMP, cell suicide as a BCL-2 family business. *Cell Death and Differentiation* 2018; **25**: 46–55.

37. Pihán P, Carreras-Sureda A, Hetz C. BCL-2 family: Integrating stress responses at the ER to control cell demise. *Cell Death and Differentiation* 2017; **24**: 1478–87.

38. Strasser A, Vaux DL. Viewing BCL2 and cell death control from an evolutionary perspective. *Cell Death and Differentiation*, 2018.

39. Ahvazi B, Boeshans KM, Idler W, Baxa U, Steinert PM. Roles of calcium ions in the activation and activity of the transglutaminase 3 enzyme. *Journal of Biological Chemistry* 2003; **278**: 23834–41.

40. Ikram MS, Neill GW, Regl G, Eichberger T, Frischauf AM, Aberger F et al. GLI2 is expressed in normal human epidermis and BCC and induces GLI1 expression by binding to its promoter. *Journal of Investigative Dermatology* 2004; **122**: 1503–9.

41. Winklmayr M, Schmid C, Laner-Plamberger S, Kaser A, Aberger F, Eichberger T et al. Non-consensus GLI binding sites in Hedgehog target gene regulation. *BMC Molecular Biology* 2010; **11**.

42. Laner-Plamberger S, Kaser A, Paulischta M, Hauser-Kronberger C, Eichberger T, Frischauf AM. Cooperation between GLI and JUN enhances transcription of JUN and selected GLI target genes. *Oncogene* 2009; **28**: 1639–51.

43. Sunjaya AP, Sunjaya AF, Tan ST. The Use of BEREPA Immunohistochemistry Staining for Detection of Basal Cell Carcinoma. *Journal of Skin Cancer*, 2017.

FIGURE LEGENDS

Figure 1 TG3 is differently expressed in skin cancer

(A) H&E staining and immunohistochemical analysis of Ki67 and TG3 expression in the normal skin ($n=10$) from TMA. **(B)** H&E staining and immunohistochemical analysis of Ki67 and TG3 expression in the cutaneous melanoma samples ($n=10$) from TMA. **(C)** H&E staining and immunohistochemical analysis of Ki67 and TG3 expression in the cutaneous squamous cell carcinoma ($n=39$) from TMA. Two representative cases of poorly- and well-differentiated tumours are shown. **(D)** H&E staining and immunohistochemical analysis of Ki67, TG3, and Ep-CAM expression in the cutaneous basal cell carcinoma ($n=13$) from TMA. Two representative cases with low and high TG3 expression are shown.

Figure 2 TG3 is highly expressed in Ep-CAM+ basal cell carcinoma

(A) H&E staining and immunohistochemical analysis of Ki67, TG3, and Ep-CAM expression in the cutaneous basal cell carcinoma ($n=6$). **(B)** Table showing the median H-score for TG3 and fold change enrichment of TG3 expression in tumour samples respect to normal skin. **(C)** Violin plot showing TG3 H-score distribution in the samples of normal skin and skin cancer from **B**.

Figure 3 *TGM3* is uniquely overexpressed in BCC respect to other TGs and its substrates

(A) Violin plot showing relative mRNA expression level of *TGM3* in normal and skin cancer samples from GSE7553. **(B)** Violin plot showing relative mRNA expression levels of *TGM1*, *TGM5*, *LOR*, and *IVL* in normal and BCC samples from GSE7553. **(C)** Immunofluorescence analysis of TG3 and LOR expression in a case of BCC. Yellow dashed line separates epidermis and dermis, white dashed lines indicate the tumour regions.

Figure 4 Differentiation-correlated profile of *TGM3* expression is lost in BCC, but not in SCC

(A) Heatmaps showing the correlation of expression between *TGM1*, *TGM3*, *TGM5*, *LOR*, and *IVL* in normal skin and different types of skin cancer from gene array GSE7553. **(B)** GO terms analysis of the top 300 genes co-expressed with either *LOR* or *TGM3* in melanoma, BCC, and SCC from gene array GSE7553. Pearson's correlation $R > +0.50$.

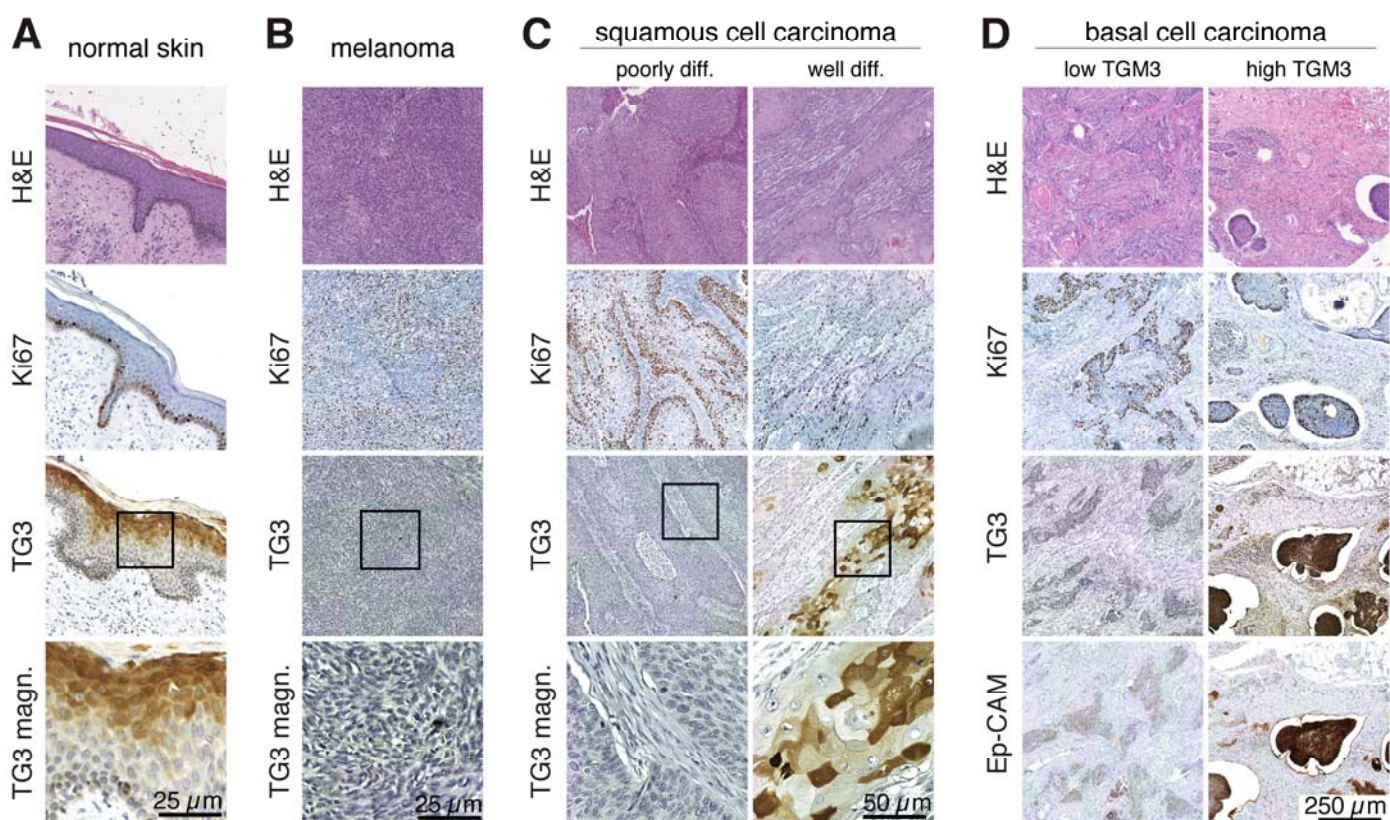


Figure 1 - Smirnov et al.

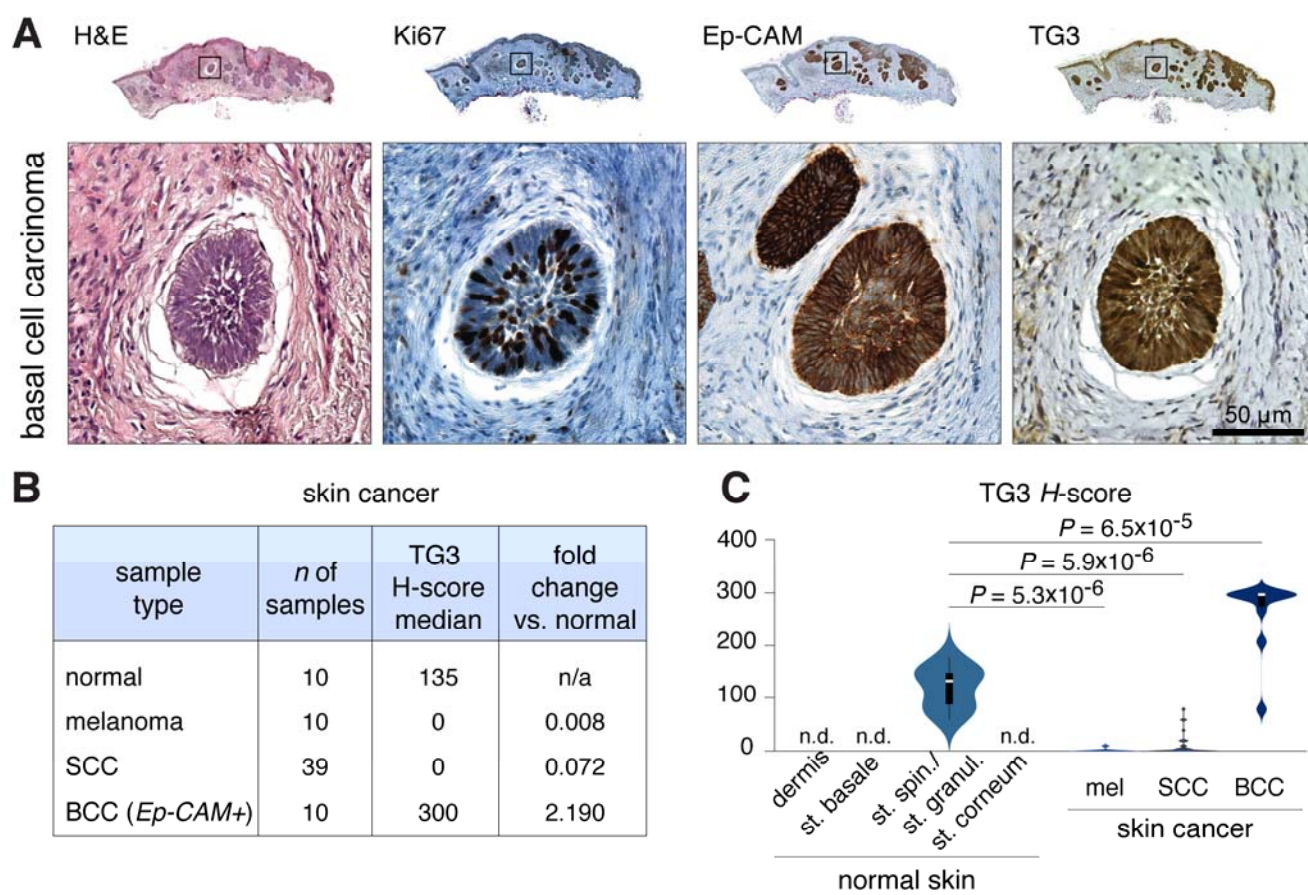


Figure 2 - Smirnov et al.

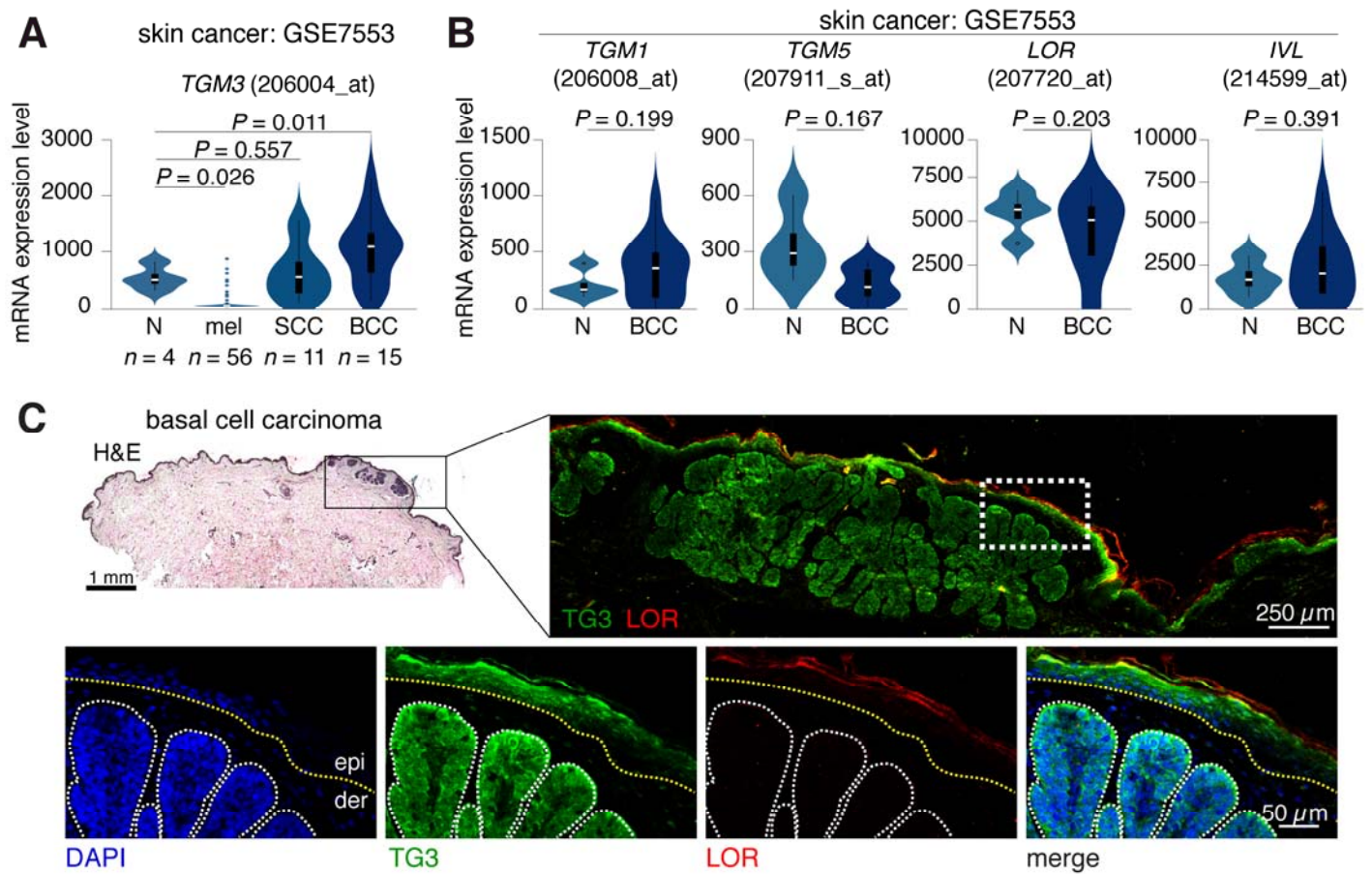


Figure 3 - Smirnov et al.

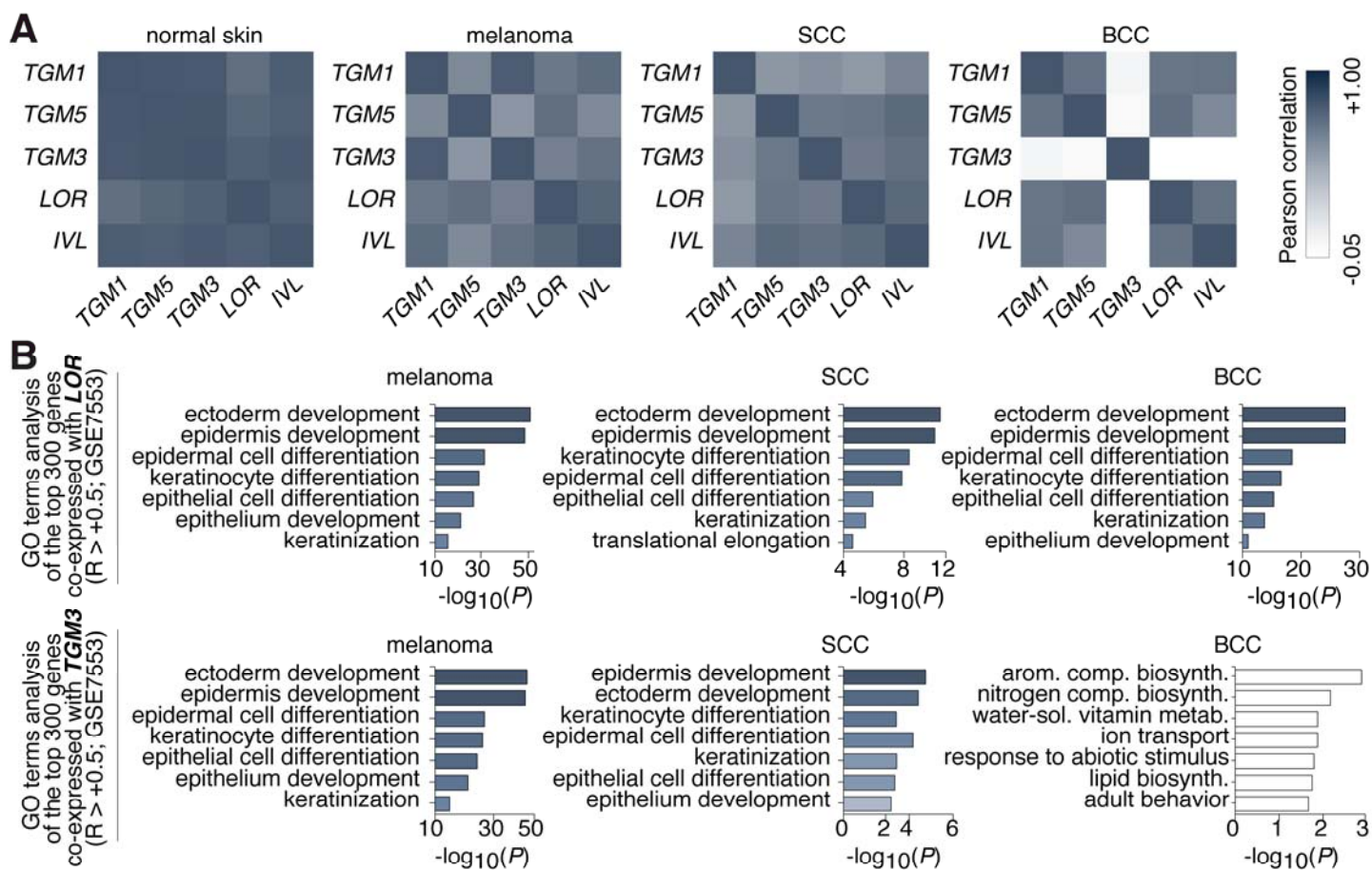


Figure 4 - Smirnov et al.

