Article:
Gao, L, van den Hurk, K, Nsengimana, J et al. (9 more authors) (2015) Prognostic Significance of Promoter Hypermethylation and Diminished Gene Expression of SYNPO2 in Melanoma. Journal of Investigative Dermatology, 135 (9). pp. 2328-2331. ISSN 0022-202X

https://doi.org/10.1038/jid.2015.163

Prognostic Significance of Promoter Hypermethylation and Diminished Gene Expression of SYNPO2 in Melanoma

Linda Gao, Karin van den Hurk, Jérémie Nsengimana, Jonathan P Laye, Joost J van den Oord, Samuel Beck, Nelleke A Gruis, Willem H Zoutman, Manon van Engeland, Julia A Newton-Bishop, Véronique J Winnepenninckx, Remco van Doorn

Cite this article as: Linda Gao, Karin van den Hurk, Jérémie Nsengimana, Jonathan P Laye, Joost J van den Oord, Samuel Beck, Nelleke A Gruis, Willem H Zoutman, Manon van Engeland, Julia A Newton-Bishop, Véronique J Winnepenninckx, Remco van Doorn, Prognostic Significance of Promoter Hypermethylation and Diminished Gene Expression of SYNPO2 in Melanoma, *Journal of Investigative Dermatology* accepted article preview 28 April 2015; doi: 10.1038/jid.2015.163.

Accepted article preview online 28 April 2015

Prognostic significance of promoter hypermethylation and diminished gene expression of SYNPO2 in melanoma

Linda Gao[1*], Karin van den Hurk[2*], Jérémie Nsengimana[3*], Jonathan P. Laye[3], Joost J. van den Oord[4], Samuel Beck[5], Nelleke A. Gruis[1], Willem H. Zoutman[1], Manon van Engeland[2], Julia A. Newton-Bishop[3], Véronique J. Winnepenninckx[2], Remco van Doorn[1]

[1] Department of Dermatology, Leiden University Medical Center, Leiden, The Netherlands.

[2] Department of Pathology, GROW-School for Oncology and Developmental Biology, Maastricht University Medical Center, Maastricht, The Netherlands.

[3] Section of Epidemiology and Biostatistics, Leeds Institute of Cancer and Pathology, University of Leeds, Leeds, UK

[4] Laboratory of Translational Cell and Tissue Research and University Hospitals, University of Leuven, KUL, Leuven, Belgium.

[5] Leiden Cytology and Pathology Laboratory, Rijswijk, The Netherlands.

*These authors contributed equally to this work.

Corresponding Author: R. van Doorn

Address: Albinusdreef 2, 2300 RC Leiden, The Netherlands

Telephone: +31-715262421

Fax number: +31-715248106

E-mail address: rvandoorn@lumc.nl

Keywords: SYNPO2, myopodin, promoter hypermethylation, melanoma, prognosis, epigenetic biomarker

Short title: SYNPO2 methylation in metastatic melanoma

Abbreviations: SYNPO2, synaptopodin 2; MCHR1, melanin-concentrating hormone receptor 1; C1orf106, chromosome 1 open reading frame 106; HIST1H3G, histone cluster 1 H3g; ZNF35, zinc finger protein 35; GNMT, glycine N-methyltransferase; FFPE, formalin-fixed paraffin-embedded; BMCA, bisulphite melting curve analysis; MSP, methylation-specific polymerase chain reaction.

Once cutaneous melanoma metastasizes prognosis of patients is generally poor in spite of recent therapeutic advances. Prognostic biomarkers are needed to better identify those patients with primary melanoma who are at increased risk of metastatic disease. Gene expression signatures have been defined that are associated with metastatic capacity of primary melanoma and survival (Winnepenninckx et al., 2006; Conway et al., 2009). Genomic characterization has revealed diverse recurrent genetic alterations including those that drive the tumorigenic process. However, the underlying molecular changes that confer the capacity to melanoma cells to migrate and colonize distant body sites remain to be defined. Promoter CpG island hypermethylation constitutes a mechanism responsible for deregulated expression of genes involved in metastasis. We and others have identified epigenetic alterations in melanoma affecting genes with a potential role in tumour cell dissemination including CDH11 and SERPINB5 (Carmona et al., 2012; Dahl et al., 2015; Gao et al., 2013). Additionally a 17-gene methylation signature was found to predict disease course of stage IIIC melanoma patients (Sigalotti et al., 2012). As epigenetic biomarker for metastasis, promoter hypermethylation has the advantage that it is stable and can be reliably detected in clinical samples. The purpose of this study was to identify methylation events that can predict development of lethal metastatic disease in patients with primary cutaneous melanoma.

To this end, we collected clinical follow-up data of 24 patients with primary melanoma whose tumours had been previously subjected to genome-wide DNA methylation profiling using Illumina 27K arrays, analyzing methylation of 14,495 genes (Gao et al., 2013). Thirteen patients had developed distant metastases, all of whom died due to melanoma, and in 11 patients no metastatic dissemination or tumour recurrence had been detected during a follow-up period of at least six years after diagnosis. We compared methylation profiles from primary melanomas of patients who developed lethal distant organ metastasis (M+) to those from primary melanomas of patients who had not developed metastasis (M-). CpG probes with an average β-value difference

(a measure of differential DNA methylation reflecting fluorescence intensity ratios between methylated and unmethylated alleles) higher than 0.20 between M+ and M- primary melanomas were considered indicative of hypermethylation of the corresponding gene promoter region. This re-analysis yielded six candidate hypermethylated genes in melanoma with metastatic behavior (M+): MCHR1, SYNPO2, C1orf106, HIST1H3G, ZNF35, and GNMT (Figure 1a). We did not find genes hypomethylated in melanoma with metastatic behavior. Validation of these six genes in an independent series of 20 fresh-frozen M+ primary melanomas and 25 M- primary melanomas using bisulphite melting curve analysis (BMCA) showed promoter hypermethylation of MCHR1 and SYNPO2 to be associated with metastatic behavior (Supplementary Table S1). We proceeded with promoter methylation analysis of MCHR1 and SYNPO2 in an independent series of 113 formalin-fixed paraffin-embedded (FFPE) primary invasive cutaneous melanoma samples with available melanoma-specific survival data using methylation-specific PCR (Supplementary Table S2a-c and Supplementary Materials and Methods). This demonstrated for the MCHR1 promoter a gradual, but not absolute methylation difference between metastatic and non-metastatic melanoma samples, limiting its use as prognostic biomarker. There was a marked difference in SYNPO2 promoter methylation between metastatic and non-metastatic melanomas (Figure 1b). Primary melanomas with SYNPO2 promoter hypermethylation had a significantly shorter survival (hazards ratio for melanoma-related death 2.01, 95% confidence interval 1.06-3.82; $P<0.034$ in univariate analysis) (Figure 2a). Importantly, SYNPO2 promoter hypermethylation remained a statistically significant prognostic factor after adjusting for tumour thickness (hazards ratio for melanoma-related death 2.02, 95% confidence interval 1.05-3.89, $P<0.034$ in multivariate analysis) (Supplementary Table S3). When considering tumour thickness, age (as continuous variables), gender and ulceration as covariates in multivariate

analysis, still an association of SYNPO2 methylation and reduced melanoma-specific survival was observed that did not reach statistical significance in this sample series.

SYNPO2 promoter methylation was shown to associate with transcriptional repression (Cebrian et al., 2008). Demethylation using 5-aza-2′-deoxycytidine of three SYNPO2-methylated melanoma cell lines resulted in reactivation of expression in treated cells, providing evidence for a link between promoter methylation and transcription regulation in melanoma (Figure 2b, Supplementary Figure S1). To examine if SYNPO2 gene expression levels provided prognostic information and to support a role for epigenetic silencing of this gene in acquisition of metastatic behaviour, we investigated its expression in 202 primary melanomas from the Leeds Melanoma cohort and tested correlation with melanoma-specific survival (Supplementary Table S2d) (Conway et al., 2009; Jewell et al., 2015). Illumina DASL HT12 v4 arrays were used to measure whole genome gene expression in FFPE tumours and after quantile normalisation melanoma-specific survival analysis was conducted (Supplementary Materials and Methods). Two SYNPO2 probes were available on the DASL array (ILMN_1688220 and ILMN_1730218). The association of low expression with shorter melanoma-specific survival was statistically significant for ILMN_1688220 before and after adjusting for age, gender and tumour thickness in Cox proportional hazards regression (hazards ratio for melanoma-related death 2.04, 95% confidence interval 1.08-3.85, $P<0.04$) (Figure 2c, Supplementary Table S4). The ILMN_1730218 probe showed a similar trend in association with melanoma-specific survival although it did not reach statistical significance after adjusting for these covariates (Supplementary Table S4). The gene expression data are therefore consistent with an association between SYNPO2 silencing by promoter methylation and shorter survival. SYNPO2 has multiple isoforms, which may explain a weaker association found for one of the probes (De Ganck et al., 2008). Summarizing, SYNPO2 hypermethylation and diminished expression are associated with

shorter melanoma-specific survival. Prognostic significance of SYNPO2 hypermethylation and gene expression is independent of tumour thickness.

In line with our observations in melanoma, SYNPO2 methylation was found to associate with tumor aggressiveness and poor clinical outcome in patients with bladder and colon cancer (Alvarez-Mugica et al., 2010; Esteban et al., 2012). In aggressive prostate cancer the locus on chromosome 4q26 harboring SYNPO2 is frequently deleted (Lin et al., 2001). SYNPO2 encodes myopodin, a member of synaptopodin family of actin-binding proteins that regulate cell shape and motility. In prostate cancer cells myopodin inhibits tumor growth and invasion in vitro and in vivo (Jing et al., 2004). The ability of myopodin to inhibit tumor cell migration depends on its binding to zyxin and integrin-link kinase (Yu and Luo, 2006; Yu and Luo, 2011). It is thought that myopodin primarily affects cell migration and invasion by inducing changes in actin cytoskeleton networks (Kai and Duncan, 2013). The potential involvement of myopodin in melanoma cell dissemination deserves further examination. The clinical value of SYNPO2 methylation in predicting metastatic behaviour of primary melanoma should be addressed in larger prospective studies, possibly combined with other candidate epigenetic markers.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgments

R.v.D. is supported by a Melanoma Research Alliance young investigator award. V.W. and K.v.d.H. are supported by Profileringsfonds Maastricht University Medical Center (PF=278). J.N., J.L. and J.N.B. are funded by CRUK Programme grant C588/A19167.

References

Alvarez-Mugica M, Cebrian V, Fernandez-Gomez JM et al. (2010) Myopodin methylation is associated with clinical outcome in patients with T1G3 bladder cancer. J Urol 184:1507-1513

Carmona FJ, Villanueva A, Vidal A et al. (2012) Epigenetic disruption of cadherin-11 in human cancer metastasis. J Pathol 228:230-240

Cebrian V, Alvarez M, Aleman A et al. (2008) Discovery of myopodin methylation in bladder cancer. J Pathol 216:111-119

Conway C, Mitra A, Jewell R et al. (2009) Gene expression profiling of paraffin-embedded primary melanoma using the DASL assay identifies increased osteopontin expression as predictive of reduced relapse-free survival. Clin Cancer Res 15:6939-6946

Dahl C, Abildgaard C, Riber-Hansen R et al. (2015) KIT Is a Frequent Target for Epigenetic Silencing in Cutaneous Melanoma. J Invest Dermatol 135:516-524

De Ganck A, De Corte V, Staes A et al. (2008) Multiple isoforms of the tumor suppressor myopodin are simultaneously transcribed in cancer cells. Biochem Biophys Res Commun 370:269-273

Esteban S, Moya P, Fernandez-Suarez A et al. (2012) Diagnostic and prognostic utility of methylation and protein expression patterns of myopodin in colon cancer. Tumour Biol 33:337-346

Gao L, Smit MA, van den Oord JJ et al. (2013) Genome-wide promoter methylation analysis identifies epigenetic silencing of MAPK13 in primary cutaneous melanoma. Pigment Cell Melanoma Res 26:542-554

Jewell R, Elliott F, Laye J et al. (2015) The clinicopathological and gene expression patterns associated with ulceration of primary melanoma. Pigment Cell Melanoma Res 28:94-104

Jing L, Liu L, Yu YP et al. (2004) Expression of myopodin induces suppression of tumor growth and metastasis. Am J Pathol 164:1799-1806

Kai F, Duncan R. (2013) Prostate cancer cell migration induced by myopodin isoforms is associated with formation of morphologically and biochemically distinct actin networks. FASEB J 27:5046-5058

Lin F, Yu YP, Woods J et al. (2001) Myopodin, a synaptopodin homologue, is frequently deleted in invasive prostate cancers. Am J Pathol 159:1603-1612

Sigalotti L, Covre A, Fratta E et al. (2012) Whole genome methylation profiles as independent markers of survival in stage IIIC melanoma patients. J Transl Med 10:185

Winnepenninckx V, Lazar V, Michiels S et al. (2006) Gene expression profiling of primary cutaneous melanoma and clinical outcome. J Natl Cancer Inst 98:472-482

Yu YP, Luo JH. (2006) Myopodin-mediated suppression of prostate cancer cell migration involves interaction with zyxin. Cancer Res 66:7414-7419

Yu YP, Luo JH. (2011) Phosphorylation and interaction of myopodin by integrin-link kinase lead to suppression of cell growth and motility in prostate cancer cells. Oncogene 30:4855-4863

Figure 1. Differential promoter methylation in primary melanomas with different metastatic outcome.

a. Methylation profiles of primary melanomas from patients who developed lethal distant organ metastasis (M^+, n=13) were compared to those from patients who had not developed metastasis (M^-, n=11), yielding six genes with average β-value difference higher than 0.20.

b. SYNPO2 amplification products from MSP are shown for two M^- primary melanomas and three M^+ primary melanomas. u, unmethylated; m, methylated; ctrl +, positive control (lymphocyte DNA treated with Sss1 methyltransferase); ctrl -, negative control (DNA from human umbilical vein endothelial cells); $H2O_{(1)}$, no template control for first amplification with flanking primers; $H2O_{(2)}$, no template control for second amplification with primers specific for methylated and unmethylated DNA.

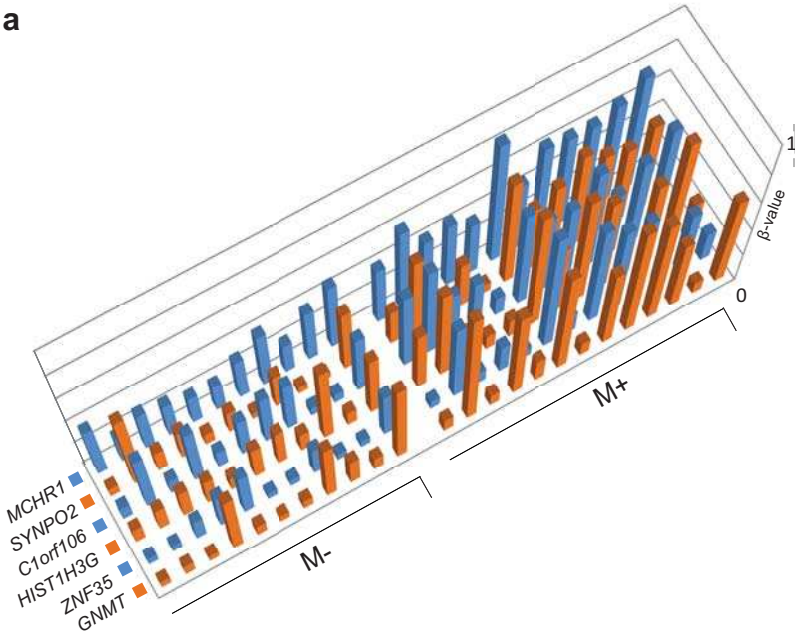
Figure 2. SYNPO2 promoter methylation and expression status are associated with survival outcome.

a. Kaplan-Meier estimate of survival function for melanoma-related death by SYNPO2 promoter methylation status was calculated and plotted against TTDOLC (time until dead or last contact). Grey line, primary melanomas scored as negative for SYNPO2 promoter methylation using MSP; black line, primary melanomas scored as positive. $P<0.05$ from Log rank test was considered significant.

b. Relative SYNPO2 mRNA expression in melanoma cell lines WM35, WM3248 and 530 treated with either mock or 5μM 5-aza-2′-deoxycytidine for 96 hours. Expression levels were analysed in duplicate. *$P < 0.05$; **$P < 0.01$.

c. Kaplan-Meier estimate of survival function for melanoma-related death by probe ILMN_1688220 measuring SYNPO2 expression in 202 primary melanomas from the Leeds

Melanoma cohort. Grey line, primary melanomas with high SYNPO2 expression; black line, primary melanomas with low SYNPO2 expression. $P<0.05$ from Log rank test was considered significant.



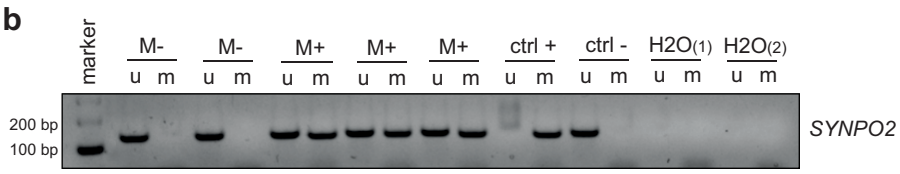
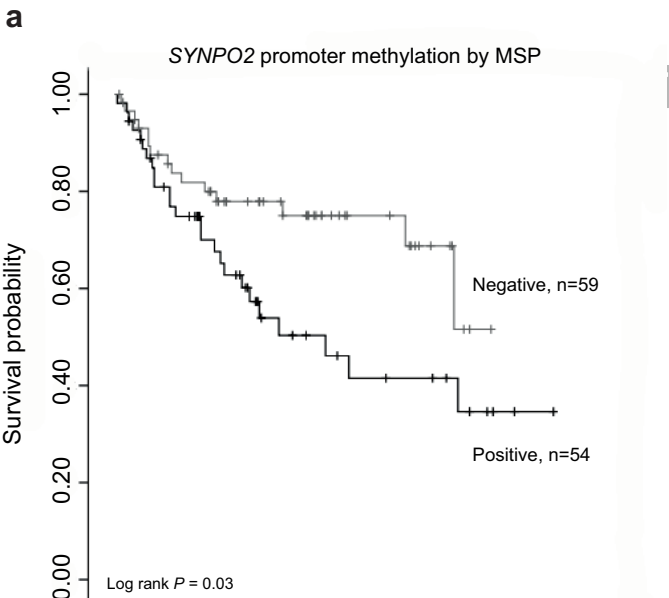
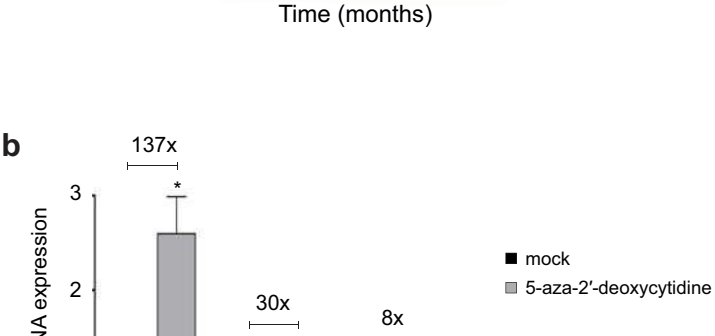


Figure 1.





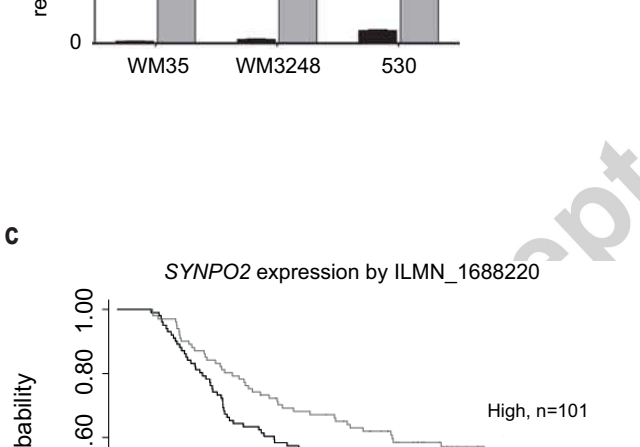


Figure 2.