Supplementary material for Limbergen E. J. V. et al. FLT1 kinase is a mediator of radioresistance and survival in head and neck squamous cell carcinoma. Acta Oncol 2014;53:637–45.

Supplementary Appendix

Primary lentivirus-based RNAi screen

The RNAi screen was conducted on the SCC61 and SQD9 HNSCC cell lines. A tyrosine kinase library based on 268 pGIPZ lentiviral shRNAmir vectors (Open Biosystems) was used. Every tyrosine kinase was targeted by multiple shRNAs. HEK293T cells were used to produce a pooled lentiviral library. HNSCC cell lines were transduced to get stable cell lines with the expression of a single shRNA. Polybrene (8 μg/ml) was added to increase transduction efficiency. Viral supernatant was removed after 24 hours. pGIPZ plasmids contained turboGFP allowing to monitor transduction efficiency using a FACSCanto flow cytometer (BD Bioscience). Cultures were split into three equal parts at day 6. That day, one part was used to extract genomic DNA (gDNA) as a reference sample, a second part was irradiated with 4 Gy of ionising radiation (Clinac, 6MV, dose rate 300 cGy/min) and cultured, whereas the third part was cultured under standard conditions. Three weeks later gDNA was harvested from the cells of the experimental conditions. ShRNA specific sequences inserted in the gDNA were PCR amplified with generic primers. PCR reactions were monitored in order to obtain PCR products from the logarithmic phase of amplification. PCR products were sequenced using a GS-FLX 454 sequencer (Roche) according to manufacturer's prescriptions and then counted using CLC genomics workbench software (CLC bio). The relative appearance of each different viral vector in the cell population was determined and compared to the reference sample. ShRNAmirs representing less than 0.1% of the total shRNA count in the reference sample were excluded from further analysis. A shRNA was carried further into the screen as a potential candidate affecting radiosensitivity if the shRNA resulted in a cell loss of at least a factor 3, and the cell loss was more pronounced in the irradiated condition as compared to the non-irradiated condition cultured in parallel. Further shRNAs that were completely lost already in the non-irradiated experimental condition were carried through as kinases affecting survival. Primarily this was done because a potential additional radiosensitising effect could not be evaluated with this screen setup as the shRNA already results in complete cell kill without radiotherapy. Additionally, these shRNAs, most strongly affecting HNSCC cell survival could reveal interesting therapeutic targets for HNSCC cancer therapy as well. Hits from a first screening round were tested again in the same setup using smaller shRNAmir pools. Consistent hits were carried through to the follow-up screen setup.

ShRNA-based follow-up screen

SQD9 and SCC61 cells were transduced with lentiviruses carrying individual shRNAmirs, causing specific expression of turboGFP in transduced cells. GFP-positive transduced cells were mixed with non-transduced cells of the corresponding cell line in order to achieve cultures containing about 50% of GFP-positive cells. As a control a non-silencing shRNAmir was used. The percentage of GFP positive cells at a given timepoint ($P_{GFP\ (TP)}$) was determined by flow cytometry. The GFP disappearing velocities (V_{GFP}) were calculated as $V_{GFP} = (P_{GFP\ (TP\ x)} - P_{GFP\ (TP\ x+1)})/\Delta t_{(TPx \rightarrow TPx+1)}$ and compared to the control condition using a one sided paired student's t-test. P-values of < 0.05 were considered significant. ShRNAs with a significant increase in V_{GFP} were considered as important for survival. To consider a hit as radiosensitising it was additionally required that the overall GFP decrease was more pronounced in the irradiated condition (1×4 Gy, Varian Clinac, 6MV, dose rate 300 cGy/min) as compared to the condition without irradiation.

Western blotting

Cells were lysed on ice with chilled cell lysis buffer (Cell Signaling) with additional Complete protease inhibitor (Roche) and sodium orthovanadate (6 mM final concentration). Proteins were separated on 4–12% NUPAGE gels (Invitrogen) and were blotted onto PVDF-membranes (Biorad). Membranes were blocked with 5% (w/v) non fatty milk in 1X TBS containing 0.2% Tween-20. Following primary antibodies were used: anti-β-Actin (#4967) and anti-KDR (#2479) (Cell Signaling technology), anti-FLT1 (ab2350) (Abcam) and anti-phospho-tyrosine (4g10) (Millipore). HRP-linked secondary antibodies were used. Detections were performed with an ECL detection system (Perkin Elmer) using an ECL imager (LAS-3000 mini). For FLT1 immunoprecipitation the FLT1 antibody (ab2350) (Abcam) was covalently coupled to Dynabeads (Invitrogen) according to the manufacturers protocol.

Immunohistochemistry and cytochemistry

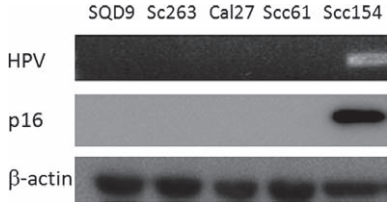
Formalin-fixed paraffin embedded tumour samples were obtained from patients from surgically resected HNSCC tumours (larynx). Four micrometer sections were dewaxed, rehydrated and peroxidase blocked according to standard protocols, followed by an antigen unmasking procedure using citrate buffer (FLT1-VEGFB) or high pH target retrieval solution (VEGFA) obtained from DAKO. To minimize aspecific immunoreactivity a blocking step was performed with protein block solution (X0909, DAKO). The following primary antibodies were used: anti-FLT1 (1:200; Atlas antibodies, HPA011740); anti-VEGFA (1:100; SC-152, Santa Cruz); anti-VEGFB (1:20; MAB751, R&D systems). Secondary antibody detection was performed with biotinylated goat anti-polyvalent antibody together with HRP-linked streptavidin (Thermo Scientific) for FLT1 and VEGFB. Envision (DAKO) was used for VEGFA. Diaminobenzidine complex (DAB) was used as chromogen. Primary antibodies were omitted in the negative controls. Counterstaining was performed with hematoxylin. For FLT1 immunocytochemistry, cells cultured on autoclaved glass coverslips were fixed in 6% formalin for 15 minutes. Then cells were permeabilised with PBS-tween for 2×5 minutes unless otherwise stated. From the blocking step on, a similar procedure was followed as the immunohistochemistry protocol. For immunocytofluorescence the secondary staining was performed with an Alexa-488 coupled Fab fragment (1/1000, Cell signaling) and counterstaining of the nucleus and cytoskeleton was done with TO-PRO3 (1μM, Invitrogen) and Alexa-555 coupled phalloidin (165nM, Invitrogen), respectively. Fluorescent images were acquired using a Leica SP5 laser scanning confocal microscope. The specificity of the FLT1 antibody HPA011740 on formalin-fixed material was confirmed by immunocytochemistry on FLT1 siRNA-treated SQD9 cells (Figure 2A–C).

Supplementary Table I.

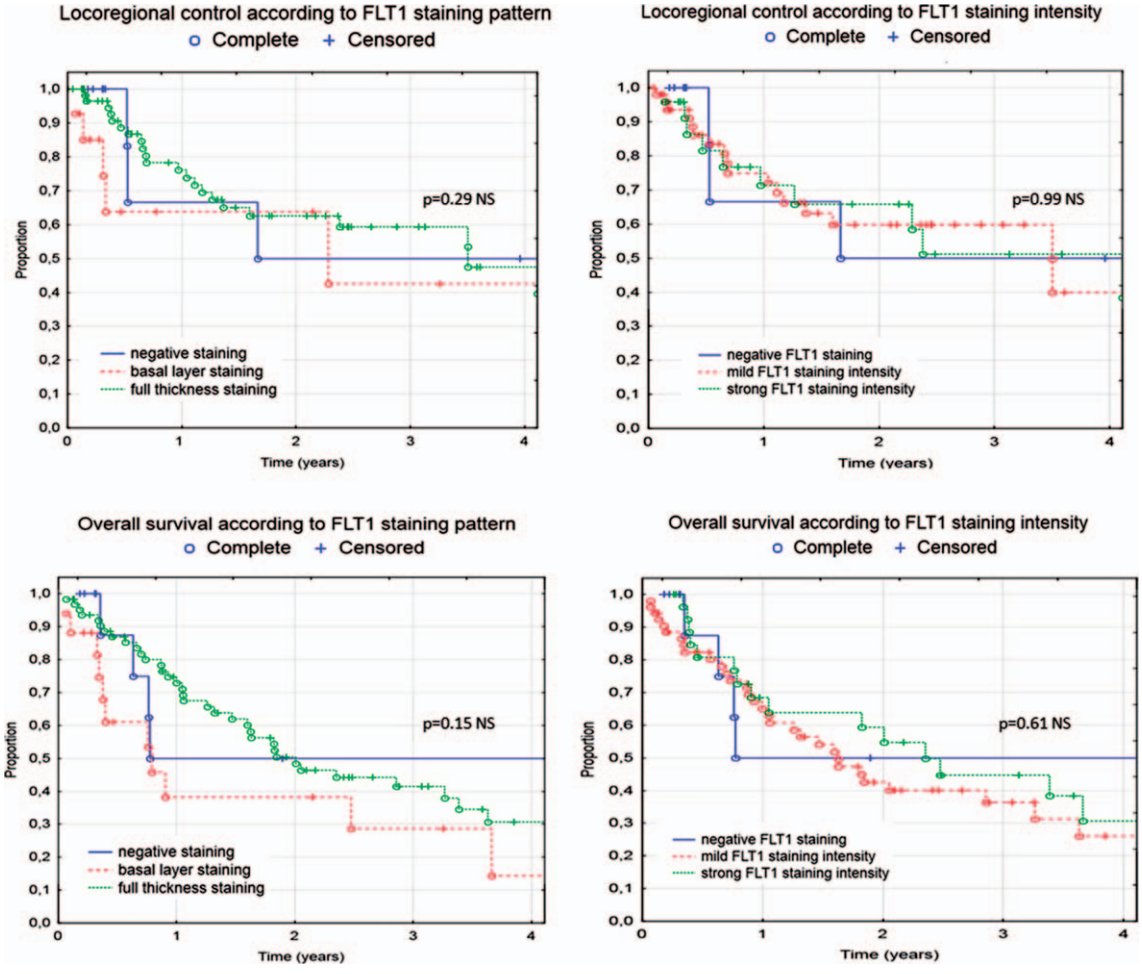
Gene	Primer sequence
FLT1*	**F:** 5′-TTGCTGTGGGAAATCTTCTCCTTA-3′
	R: 5′-TGCCTTCCCTCAGGCGA-3′
VEGFA	**F:** 5′-CCATGCAGATTATGCGGATCA-3′
	R: 5′-GCATTCACATTTGTTGTGCTGTA-3′
VEGFB	**F:** 5′-TCCCTGAGGCCATCATCAA-3′
	R: 5′-CTGAGCTGGTATGTGACCC-3′
PlGF	**F:** 5′-CGTCGTGTCCGAGTACC-3′
	R: 5′-AGTGCAGATTCTCATCGCC-3′
KDR	**F:** 5′-TCCCTGCGAAGTACCTTGGTTA-3′
	R: 5′-GTGTCTCTTTCACTCACTTCCATA-3′
HPRT	**F:** 5′-TGACACTGGCAAAACAATGCA-3′
	R: 5′-GGTCCTTTTCACCAGCAAGCT-3′

The PCR cycle conditions were as follows: a preincubation step for 10 minutes at 95°C followed by 40 amplification cycles; each cycle included 10 seconds at 95°C and 30 seconds at 60°C; followed by melting curve analysis to verify specific amplification of the amplified product.

*The primer set of FLT1 was taken over exons 24-25, thereby omitting possible detection of the FLT1 splice variants without tyrosine kinase domain [cfr. Sela S, et al. Circ Res 2008;102: 1566–74].



Supplementary Figure 1. HPV status of the different cell lines was determined by GP5+/6+PCR [cfr. de Roda Husman AM, et al. J Gen Virol 1995]. P16 Western blotting was used as a confirmation. Only SCC154 is HPV related. B-actin is used as loading control.



Supplementary Figure 2. Locoregional control and overall survival in patients with oropharyngeal squamous cell carcinoma of the head and neck, treated with radiotherapy, according to immunohistochemical FLT1 staining pattern or staining intensity. Data are presented as Kaplan-Meier survival curves.