Int. J. Cancer: **117,** 723–729 (2005)

Activating mutations in the extracellular domain of the melanoma inducing receptor Xmrk are tumorigenic *in vivo*

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Mutated versions or overexpression of receptor tyrosine kinases such as the epidermal growth factor receptor are found frequently in various cancers. In Xiphophorus the formation of hereditary melanoma is caused by the overexpression of the *Xmrk* oncogene locus. Xmrk is a mutationally altered version of the epidermal growth factor receptor. Two amino acid changes in the extracellular domain of the receptor were shown *in vitro* to be responsible for a constitutive, ligand-independent activity of Xmrk. To analyze whether these two mutations are indeed responsible for the *in vivo* oncogenic activity of the receptor, both were independently introduced into the wild-type, non-oncogenic Xiphophorus EGF-receptor and tested in Medaka embryos for their tumorigenic capacity. Both mutations were sufficient to induce tumors after short latency periods and at a comparable frequency as the native *Xmrk* oncogene. The G359R mutation led to a significantly higher tumor rate than the C578S mutation. Our study shows that subtle point mutations of the EGF-receptor can lead to a highly tumorigenic oncoprotein.

Key words: epidermal growth factor receptor; Xmrk; embryonic tumors; Medaka

Receptor tyrosine kinases (RTK) are important regulators of cell development and growth. Consequently, impairment of their normal function is often connected to pathological conditions. In particular a variety of human neoplasms are characterized by quantitative or qualitative alterations of RTK.[1,2] Members of the human epidermal growth factor receptor (EGFR) family of RTK are frequently found to be constitutively activated.[3,4] Multiple ways of deregulation of RTK are discussed for melanoma based on the observation that melanocytes become growth factor independent during progression to malignant melanoma cells.[5,6] For studying the function of EGFR-related RTK in tumorigenesis in general and melanoma in particular, melanoma formation in hybrids of the fish Xiphophorus provides a useful animal model. The tumors arise due to overexpression of the RTK Xmrk. Xmrk is an oncogenic version of the epidermal growth factor receptor in Xiphophorus. The *Xmrk* oncogene arose by a gene duplication event from the Xiphophorus *egfrb* proto-oncogene.[7–10] In the parental, healthy fish *Xmrk* is not expressed at sufficient levels to induce tumors. This is due to the presence of an unlinked locus (designated *R*, also known as *Diff*) in the genome of *Xmrk* carrying fish that suppresses the transcription of the oncogene. In specific crosses, however, the chromosomes harboring the *R* locus are substituted by chromosomes from the crossing partner that do not contain this locus. This results in the overexpression of *Xmrk* in the pigment cell lineage and consequently in the development of malignant melanoma in the hybrids.[11–14]

Studies on the signal transduction of the *Xmrk* gene product in several cell culture systems demonstrated that the encoded RTK is sufficient to induce several alterations that characterize the neoplastically transformed phenotype, namely a strong stimulation of cell proliferation and anti-apoptotic signaling,[15,16] inhibition of differentiation[17] and survival of melanocytes in the dermis.[18] *Xmrk*, when introduced as a transgene into cell lines or fish embryos is able to transform a whole variety of cell types other than pigment cells, demonstrating its strong oncogenic potential.[18,19] The restriction to melanoma in the *in vivo* situation is apparently due to the fact that in the hybrid genotypes *Xmrk* is only expressed at sufficient levels in this cell lineage.

Ectopic expression of chimeric versions of the Xmrk receptor in various cell lines showed that the receptor is constitutively activated in a ligand-independent manner.[20,21] Further studies showed that the constitutive activity of the receptor *in vitro*, apparent by the high degree of tyrosine autophosphorylation, is due to two of 15 amino acid changes that are found in the oncogene product compared to the proto-oncogene encoded egfrb protein. Both mutations are located in the extracellular domain of the receptor. One (C578S) is a cysteine that is mutated in the Xmrk protein to a serine at position 578, the other (G359R) is a glycine to arginine exchange at position 359. Under non-reducing conditions, when disulfide bridges are intact, Xmrk forms a dimer. This dimer formation was also observed for proto-oncogene encoded Egfrb proteins into which the G359R or C578S mutations were introduced.[22] It was thus reasoned that these two mutations induce covalent dimer formation of the Xmrk receptor. This would lead to ligand independent autophosphorylation and consequently constitutive signaling of the receptor as seen in cells expressing the mutant receptors. It was not clear, however, if the activating mutations identified by *in vitro* studies will be oncogenic *in vivo*. To analyze whether the two amino acid changes are responsible for the oncogenic activity of Xmrk *in vivo*, DNA constructs encoding the proto-oncogenic form of Egfrb and mutated version were injected in fish embryos. We found that both mutations independently confer a tumor inducing activity to Egfrb confirming the biochemical model for the oncogenic activation of this receptor tyrosine kinase.

Material and methods

Expression plasmids

For control a *CMV::egfp* construct (earlier designated pCS2-GFP) was used.[23] For expression of the proto-oncogene the *egfrb* cDNA (earlier designated INV) with the CMV enhancer and Tk promoter was used.[21] To generate *CMV-Tk::egfrb* with the C578S mutation the 1238 bp *Sbf1–Sgr*A1 fragment was replaced by the corresponding fragment from prk5-INV-CS,[22] which contains the C578S mutation. To generate *CMV-Tk::egfrb* with the G359R mutation the 1238 bp *Sbf1–Sgr*A1 fragment was replaced by the corresponding fragment from prk5-INV-GR,[22] containing the G359R mutation. To verify the correctness of the cloning procedures the expression plasmids were partially sequenced using the CEQ™ DTCS dye terminator cycle sequencing kit and the CEQ 2000XL DNA Sequencer (Beckman-Coulter). For comparison the expression vector *CMV::Y-ONC* was used, which contains a native oncogenic Y-chromosomal *Xmrk* allele of *X. maculatus*.[19]

Grant sponsor: Deutsche Forschungsgemeinschaft; Grant sponsor: Fonds der Chemischen Industrie.

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Received 21 December 2004; Accepted after revision 11 March 2005
DOI 10.1002/ijc.21232
Published online 14 June 2005 in Wiley InterScience (www.interscience.wiley.com).

Cell culture, DNA transfections and Western blotting

HEK 293T cells (human embryonic kidney fibroblasts with SV40 T-antigen) were grown in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS, 1% glutamine and antibiotics. Expression vectors were transiently transfected into HEK 293T cells by the calcium-phosphate method.[24] Cell lysis and Western blotting were done essentially as described.[16] Anti-pep-mrk[25] and anti-Ptyr[26] antibodies were used to detect the Egfrb proteins.

Embryos

Medakafish (Oryzias latipes; Teleostei: Cyprinodontidae) were purchased from Carolina Biological Supply Company (Burlington, NC) in 1988 and kept for breeding at the Max-Plank-Institute of Biochemistry (Martinsried, Germany) and since 1991 at the Department of Physiological Chemistry I, University of Wuerzburg. Adult fish were maintained under standard conditions[27] with an artificial photoperiod (14:10 hr light:dark) to induce reproductive activities. Clusters of fertilized eggs were collected 0.5–1 hr after the onset of light and kept in a rearing medium containing 0.1% NaCl, 0.003% KCl, 0.004% $CaCl_2 \times 2H_2O$, 0.016% $MgSO_4 \times 7H_2O$ and 0.0001% methylene blue. Embryos were staged according to Iwamatsu.[28]

Microinjection

DNA was injected into the cytoplasm of one cell of the 2-cell stage Medaka embryo exactly as described earlier.[29] Approximately 500 pl (equivalent to 25 pg) of plasmid DNA were injected into each embryo. DNA was dissolved to a final concentration of 50 μg ml^{-1} in deionized water. Plasmid-DNA was injected in supercoiled conformation. After injection the embryos were kept at 26°C in a Petri dish (9 cm in diameter) filled with the fish-medium described above. For a period of 18–22 days they were observed daily under a stereo-microscope. Dead embryos were removed to avoid bacterial contamination. The hatched young fish were kept in larger dishes (14.5 cm in diameter) at room temperature. They were fed once a day. For observation they were anesthetized with 0.05% 2-phenoxyethanol.

Histology

For histological analysis of tumors and tumor-like lesions embryos and young fish were fixed for 2–4 days in Bouin's fixative (saturated picric acid in H_2O:formaldehyde [37%]:glacial acidic acid, 15:5:1), transferred to fixative solution (1 × PBS [10%], formaldehyde [3%]) and finally embedded in paraffin. Sections (5 μm) were stained with H&E.

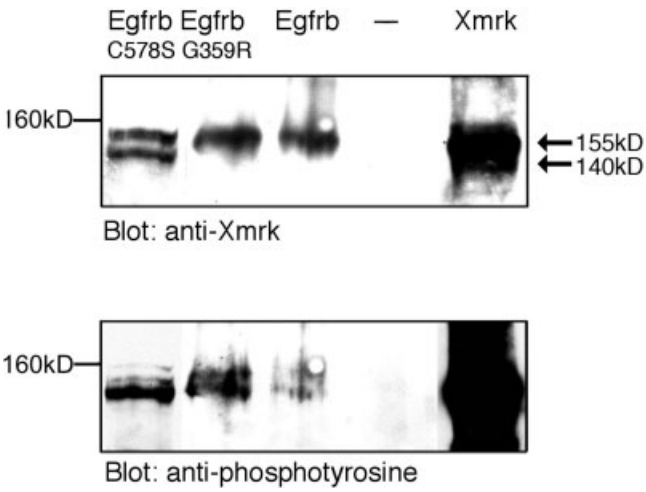

FIGURE 1 – Western blot analysis of whole cell lysates from HEK 293T cells transfected with different *egfrb* constructs: Egfrb C578S, CMVTK::egfrb-C578S; Egfrb G359R, CMVTK::egfrb-G359R; Egfrb, CMVTK::egfrb; Xmrk, CMV::Y-Onc. The expression level of the Xmrk receptor was analyzed using polyclonal anti pep-mrk. Receptor phosphorylation was analyzed using an anti-phosphotyrosine antibody. The 140 kD band corresponds to aberrant glycosylation of the mutated receptors.

Results

The mutations G359R and C578S when introduced in the proto-oncogenic Egfrb receptors of Xiphophorus produce constitutively active dimers *in vitro* but it was unknown if these mutations are sufficient to produce an oncogenic receptor *in vivo* and to induce tumors. For studying the activity of the mutant Egfrb receptors *in vivo* they were expressed from the Tk promoter containing a CMV enhancer region, because this promoter was shown to be most efficient for *in vivo* expression in embryonic fish.[30] Before the *in vivo* experiments the expression vectors (*egfrb* G359R and *egfrb* C578S) were tested *in vitro*. After transient transfection in 293T cells proteins of the expected size of 155 kD were detected with an Xmrk specific antibody (Fig. 1, *upper panel*). The presence of the 140 kD band in the C578S mutant as well as in Xmrk can be explained by aberrant glycosylation of the proteins as a result of the mutation.[22] Reprobing of the membrane with an anti-phosphotyrosine antibody showed that both mutant proteins were strongly auto-phosphorylated (Fig. 1, *lower panel*). By contrast, wild-type Egfrb showed only a weak auto-phosphorylation, whereas the native oncogenic Xmrk was expressed as a highly phosphorylated protein.

To assess the oncogenic potential of the two Xmrk receptor mutants G359R and C578S *in vivo*, their capacity to induce tumor formation in Medaka fish was analyzed and compared to that of the proto-oncogenic receptor. As a control for effects induced by the microinjection of the embryos 143 embryos were injected with the *CMV::egfp* construct. A total of 128 embryos (90%) showed a clearly visible GFP expression. As a typical phenomenon of transient expression of DNA injection into early fish embryos[29,30] the intensity of GFP fluorescence and the pattern of GFP expression varied between different embryos and was mosaic.

None of the embryos developed any sign of hyperproliferation or of neoplastic lesions. The lethality rate was 6.3%. 10% of the injected fish developed deformations during the early development (Table I).

TABLE I – LETHALITY, DEFORMATIONS AND TUMOROUS LESIONS AFTER ECTOPIC EXPRESSION OF DIFFERENT *egfrb* CONSTRUCTS

DNA construct	Injected embryos/embryos survived Stage 28 [*n*]	Early embryonic lethals[1] [*n*] (%)	Deformed embryos[2] [*n*] (%)	Tumorous lesions[3] [*n*] (%)	Fraction of epithelial hyperplasia/cysts [*n*] (%)
CMV::eGFP	143/134	9 (6.3)	15 (10.5)	0	0
CMVTK::egfrb	202/171	31 (15.3)	35 (17.3)	4 (2.3)	4 (100)
CMVTK::egfrb-C578S	347/260	87 (25.1)	64 (18.4)	25 (9.6)	16 (64)
CMVTK::egfrb-G359R	337/222	115 (34.1)	62 (18.4)	42 (18.9)	21 (50)
CMV::Y-Onc	264/136	128 (48.5)	40 (15.2)	20 (14.7)	13 (65)

[1]Up to Stage 28.–[2]Up to Stage 28, these embryos never passed to hatching stage.–[3]Percentage calculated as embryos with tumorous lesions/total number of embryos that developed beyond Stage 28 = survived Stage 28.

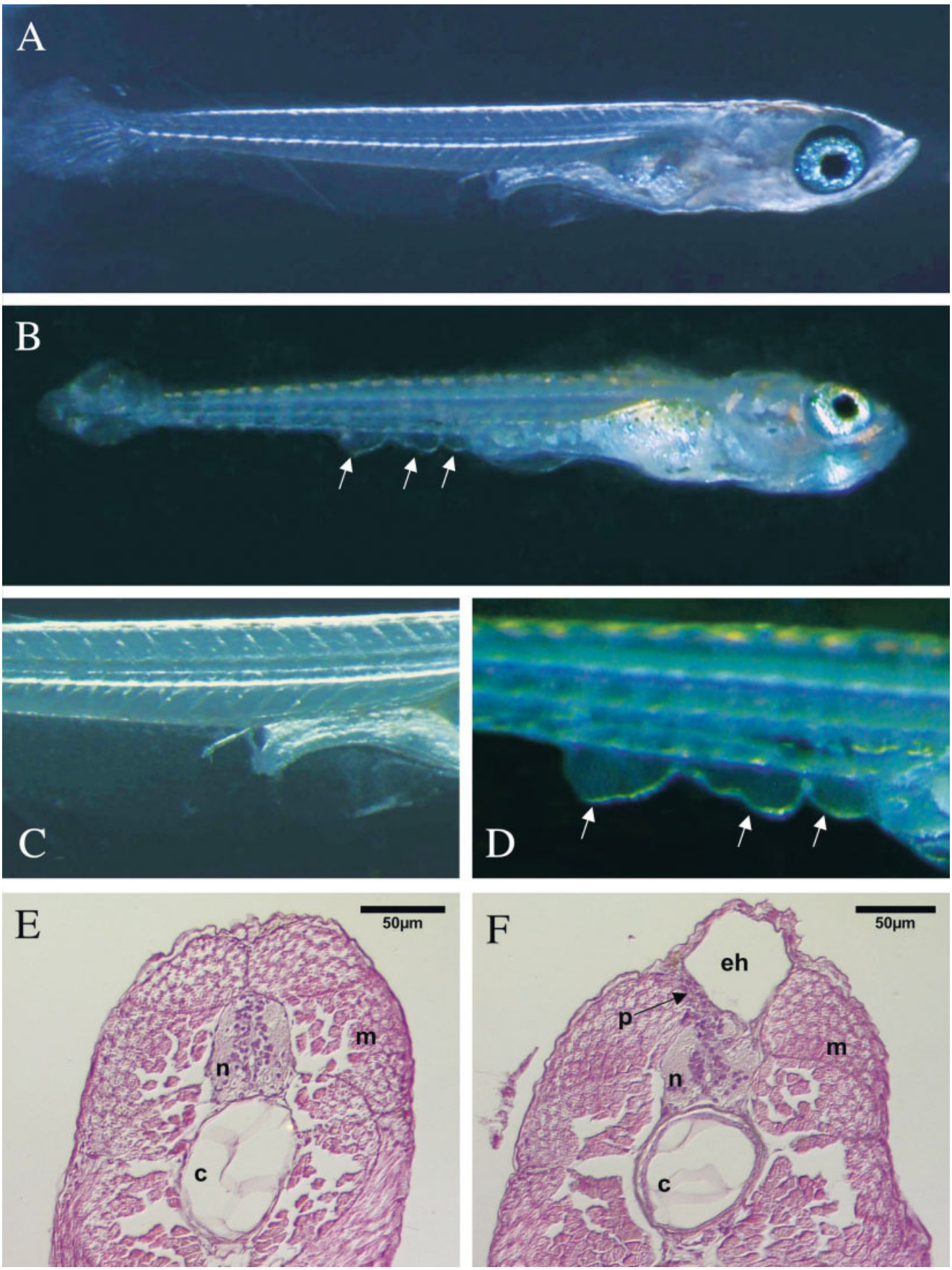

Figure 2 – (*a*) Fourteen-day-old uninjected Medaka fish. (*b*) Fourteen-day-old fish injected with CMVTK::egfrb that developed cysts (arrows). (*c*) Magnification of (*a*). (*d*) Magnification of (*b*). (*e*) Histological section of an 11-day-old control fish (uninjected). (*f*) Section of an 11-day-old fish (expressing CMVTK::egfrb-C578S) with a cyst. c, chorda dorsalis; n, neural tube; m, muscle; p, proliferating cells; eh, epithelial hyperproliferation (cyst).

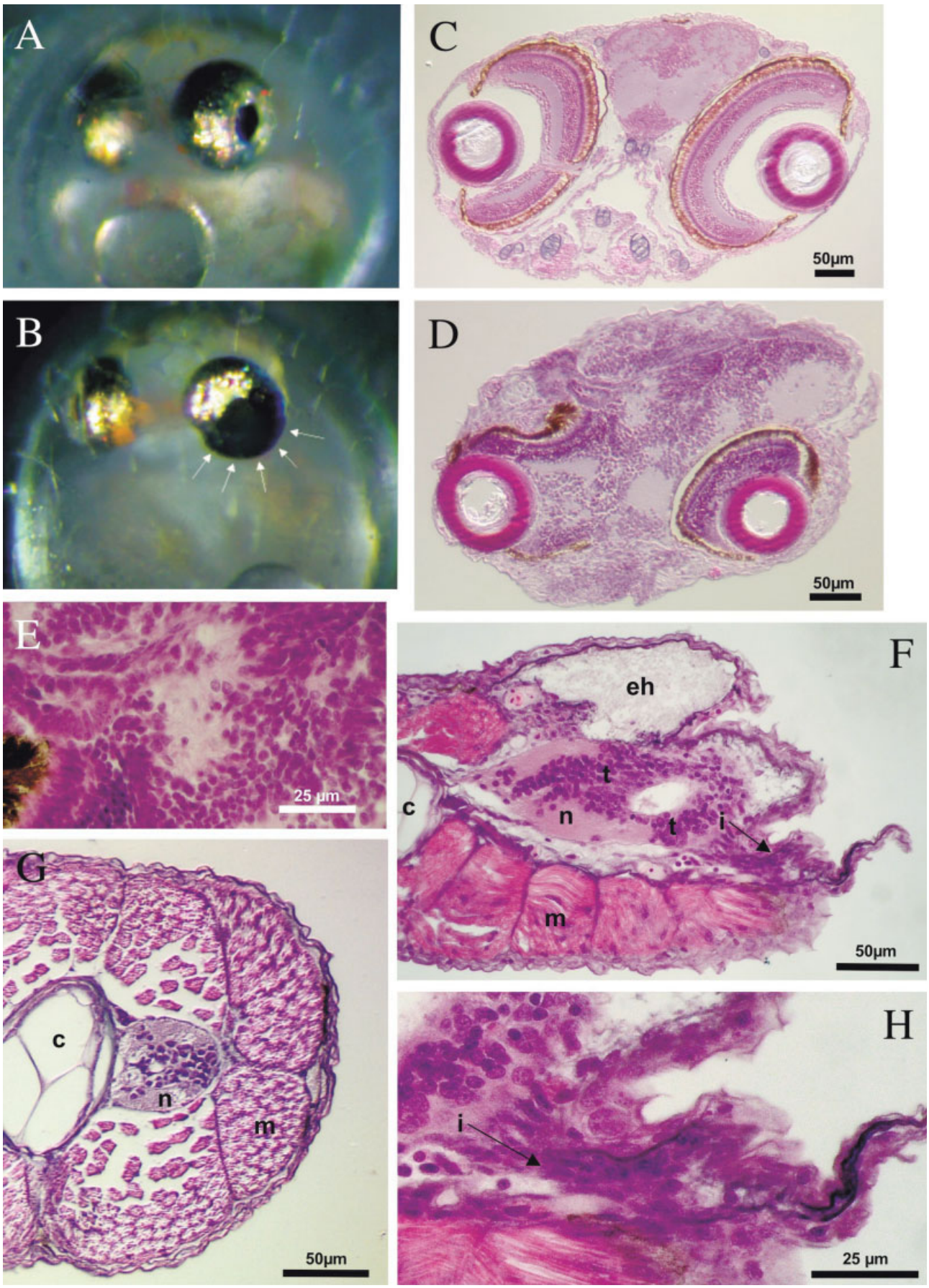

FIGURE 3.

For the analysis of the overexpression of the proto-oncogene a total of 202 embryos were injected with an egfrb expression construct (*CMV-TK::egfrb*; see Table I). Only 4 of 171 embryos that developed beyond organogenesis (Stage 28) developed tumorous lesions after a latency period of approximately 10–12 days. All were classified as epithelial hyperproliferation, which became apparent as epidermal cysts (Fig. 2). These lesions never grew out to solid tumors or showed any sign of malignancy. The background tumor rate was 1.98% of all injected embryos or 2.3% when only embryos, which survived the first 3 days of development (Stage 28), were counted.

When even higher amounts of *egfrb* than for the other constructs (up to 10-fold, approximately 250 pg DNA, the maximum of transgene DNA that is tolerated by the Medaka embryo) were injected, also solid tumors like with the C578S, G359R and Y-Onc constructs were observed. The tumor rate was 14% (7 of 50, data not shown).

To investigate the effect of the C578S mutation *in vivo* 347 embryos were injected with the *CMV-TK::egfrb* C578S construct. Twenty-five embryos developed tumorous lesions, which corresponds to 7.2% (of all injected embryos) and 9.6% (embryos surviving to organogenesis stage, Stage 28) (Table I). 16 of these 25 tumors were classified as epithelial hyperproliferation (Fig. 2*F*). They were macroscopically detectable not earlier than between Day 9 and 18, with an average latency period of 14 days.

Four of the injected embryos showed lesions, which affected the brain and another 4 developed neoplastic growth of the eye (Fig. 3*b*). These tumors had a shorter latency period (average = 7.8 days) than the epithelial hyperproliferation. All these tumors were solid masses that became apparent as large areas of undifferentiated cells with a low cytoplasm to nuclear ratio. They destroyed the normal architecture of the developing organ in the embryo and led to tumorous outgrowth of the organ in which the neoplasia developed. Due to the generally low differentiation of cells in the embryo and due to a lack of markers, no histopathological subclassification of the tumors could be made.

For the *in vivo* analysis of the G359R mutation 337 embryos were injected with *CMV-TK::egf b* G359R. We observed the formation of tumorous lesions in 42 cases. The tumor rate was significantly higher than after injection of the C578S construct. 21 tumorous lesions were diagnosed as epithelial hyperproliferation (Table I). They developed after an average latency period of 13.6 days. The remaining 21 tumors were solid neoplasias that occurred in the developing brain, the embryonic retina and the integument (Fig. 3*d*,*e*,*g*,*h*). The latency period of these tumors again was much shorter, between 6–13 days (average = 8 days).

For a positive control and for comparison with earlier studies a construct containing a natural allele of the *Xmrk* oncogene under the control of the CMV promoter (*CMV::Y-Onc*) was injected. As expected the transgene induced the formation of tumorous lesions. 7.6% of all injected embryos and 14.7% of embryos surviving at organogenesis (Stage 28) developed tumors. Sixty-five percent of the tumors were classified as epithelial hyperproliferation with an average latency period of 12.9 days, whereas the rest were solid tumors that occurred in the developing brain, the embryonic retina and the integument. These more aggressive tumors showed an average latency period of 9.2 days.

FIGURE 3 – (*a*,*b*) Nine-day-old embryos. (*a*) Uninjected control. (*b*) Embryo expressing CMVTK::egfrb-C578S with a tumor of the eye (arrows), originating from the retinal layer. (*c*) Coronal section through the head of a 10-day-old control fish (uninjected). (*d*) Coronal section of a 10-day-old fish (expressing CMVTK::egfrb-G359R) with a tumor of neural origin that led to destruction of the retinal structures. (*e*) Magnification of (*d*). (*f*) Transversal section of a 15-day-old fish expressing CMVTK::egfrb-G359R with a tumor of the spinal cord that has invaded the adjacent muscular compartment. Note that in the tumorous sector muscle development is severely disturbed and that a cavity has formed above the lesion. (*g*) Transversal section of a 15d old control fish (uninjected). (*h*) Magnification of (*f*). c, chorda dorsalis; n, neural tube; m, muscle; t, tumor cells; eh, epithelial hyperproliferation/cysts; i, invading cells.

Discussion

Using the Medaka *in vivo* tumorigenesis assay we could show that mutated versions of the Xiphophorus EGF receptor are sufficient to induce neoplastic growth (Fig. 4). For control and comparison we injected the proto-oncogene and a natural occurring allele of the oncogene *Xmrk*. For the proto-oncogene we obtained a tumor rate of 2.3%, which is in good agreement with the tumor rate of 3.2% obtained in an earlier study using the same construct.[21] The tumor type was exactly the same, the few lesions that developed were benign epithelial hyperproliferation. This confirms that the *egfrb* gene of Xiphophorus even when expressed at the same level as *Xmrk* does not act as an oncogene. When *egfrb* is, however, highly overexpressed, tumors are induced. This is in line with findings in mammalian cancers, where overexpression of not mutationally altered EGFR family RTK is connected to tumor formation.[4]

In our study the oncogene c-DNA led to a tumor rate, which is not different from a previous study using the *CMV::Y-Onc* construct (all injected embryos: 7.9 *vs.* 8.5%, embryos that survived the first 3 days of development: 14.7 *vs.* 13.2%).[19] This demonstrates the reproducibility of the Medaka embryo tumorigenicity assay.

The C578S and the G359R mutation have been shown *in vitro* to lead to constitutive activation of the Xiphophorus EGF-receptor.[22] Our present work shows that these mutations are tumorigenic *in vivo*. Both mutations led to a high rate of tumor formation whereas the wild-type *egfrb* produced only benign cysts. In the oncogenic receptor the cysteine at position 578 is replaced by a serine. In the wild-type EGF-receptor this cysteine is involved in the formation of an intramolecular cystine-bridge. Consequently in the mutated protein the partner cysteine is now unligated and can form an intermolecular disulfide bond between two receptor monomers. The covalent dimer would mimic the conformation of ligand-bound receptors and lead to a constitutively activated receptor. The effect of the second mutation (G359R) is more difficult to explain. The small and unpolar amino acid glycine at position 359 is replaced by the big and polar amino acid arginine. It is possible that the big side chain sterically hinders the formation of a neighboring intramolecular cystine-bridge. The resulting unpaired cysteines could form intermolecular disulfide bonds between 2 receptor monomers, which would also explain why the G359R mutant protein runs as dimers on PAGE under non-reducing conditions.[22] Interestingly after injection into Medaka embryos the C578S mutation was significantly less tumorigenic than the G359R mutation. It seems as if the G359R mutation creates receptor dimers that are more active or more stable.

Mutated versions of many receptors are frequently found in human tumors. Different mutations in the extracellular and intracellular domain of the EGF-receptor, promoting constitutive kinase activity in the absence of ligand have been described.[3] Mutations of this sort have been detected in a variety of human cancers, including glioblastomas,[31] non-small cell lung carcinomas,[32] breast carcinomas[33] and others.[34] Some transgenic mouse lines have been established that express mutated versions of the EGF-receptor.[35,36] Due to the considerable efforts and time that is inevitable to produce transgenic mice, such animals have been used more to show that mutated, constitutively activated EGFR have the capacity to induce tumorous growth *in vivo*. A routine, simple and quick whole animal test for tumorigenic potential of mutations detected in human cancers is not possible. The Medaka embryo assay that we have used here might bridge this gap. Although being a lower vertebrate, the major hallmarks of EGFR-induced tumorigenesis are seen, and by injection of mutated versions within short time in a simple experiment information on the tumorigenic potential of a specific mutations can be obtained. Neutral changes and polymorphisms can be differentiated from

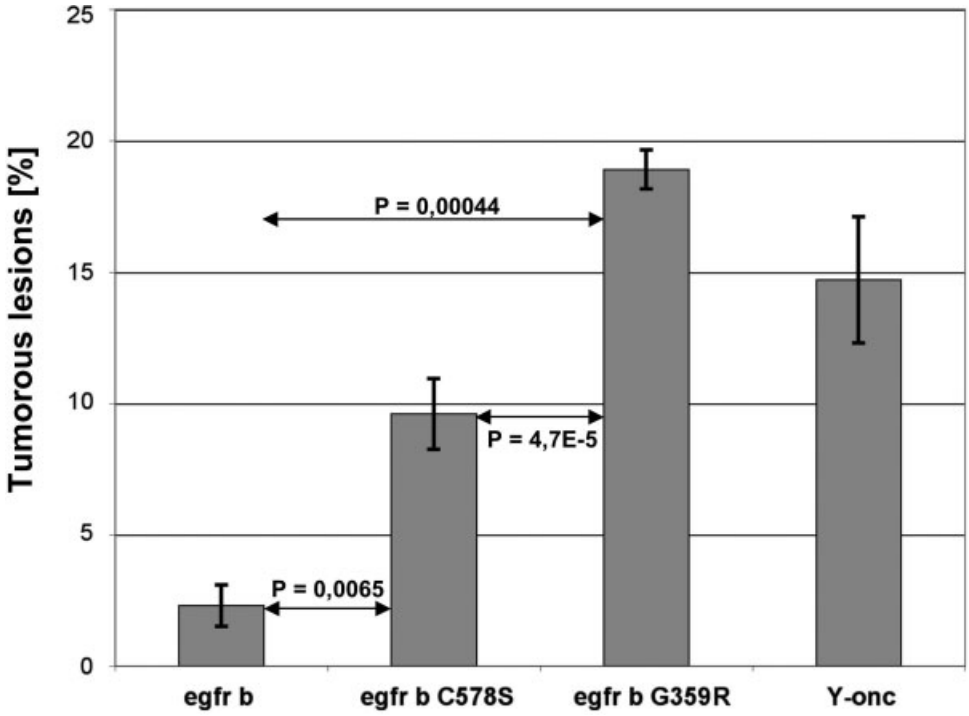

FIGURE 4 – Percentage of tumorous lesions (percentage calculated as embryos with tumorous lesions/total number of embryos that developed beyond Stage 28) recorded in Medaka embryos and young fish after the injection of different versions of the egfrb receptor. egfr b, CMVTK::egfrb (n = 202); egfrb C578S, CMVTK::egfrbC578S (n = 347); egfrb G359R, CMVTK::egfrbG359R (n = 337); Y-Onc, CMV::Y-Onc (n = 264). The p values were calculated by Mann-Whitney U-test and show that the differences in tumor frequencies are significant. Note that the p-value of egfrb/egfrb G359R is higher than egfrb C578S/egfrb G359R due to fewer injection experiments with egfrb.

tumor causing mutations. Especially in cases where the effect of an amino acid exchange cannot be predicted easily, like in the case of the G359R mutation of Xmrk, or mutations in the FGF-receptor 2 and the RET receptor of MEN2a patients, which like the G359R mutation did not affect cysteines involved in intramolecular disulfide bridges but anyway induced covalent receptor dimerization,[37,38] the Medaka tumorigenicity assay may give a quick answer. Future experiments will show whether also mutated versions of other receptors of the EGFR family, or from other RTK families are tumorigenic as well.

Acknowledgements

We thank C. Froschauer for expert assistance in the *in vitro* analysis of the expression constructs, E. Geissinger for advice in the diagnosis of the tumorous lesions, R. Wacker for the histological sections and H. Schwind for breeding of the fish. This work was supported by the Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 587 (Regulatorische Membranproteine), Transregio 17 (The ras pathway in human cancer) and Fonds der Chemischen Industrie.

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