MicroRNA-24 regulates XIAP to reduce the apoptosis threshold in cancer cells

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Abstract

MicroRNAs have been implicated as important mediators of cancer cell homeostasis, and accumulating data suggest compelling roles for them in the apoptosis pathway. X-linked inhibitor of apoptosis protein (XIAP) is a potent caspase inhibitor and an important barrier to apoptotic cell death, but the mechanisms which determine the diverse range of XIAP expression seen in cancer remains unclear. In this study, we present evidence that miR-24 directly targets the 3′UTR of the XIAP mRNA to exert translational repression. Using a heuristic algorithm of bioinformatics analysis and in vitro screening, we identified miR-24 as a candidate regulator of XIAP expression. Array CGH and SKY analysis reveal that genomic copy number loss at the miR-24 locus is concordant with loss of endogenous miR-24 in cancer cells. Using a luciferase construct of the XIAP 3′UTR, we showed that miR-24 specifically coordinates to the XIAP mRNA. And interference with miR-24’s binding of the critical seed region, resulting from site-directed mutagenesis of the 3′UTR, significantly abrogated miR-24’s effects on XIAP expression. Moreover, miR-24 over-expression can overcome apoptosis-resistance in cancer cells via down-regulation of XIAP expression, and the resulting cancer cell death induced by TRAIL is executed by the canonical caspase-mediated apoptosis pathway. In summary, our data suggest a novel mechanism by which miR-24 directly modulates XIAP expression level and consequently the apoptosis threshold in cancer cells.

Conflict of interest

The authors declare no conflict of interest.
Keywords
microRNA; cancer; XIAP

Introduction
Apoptosis is an intrinsic cell death mechanism expressed in mammalian cells, but is often defective in cancer cells (1). Maintaining homeostasis in the normal cell requires a balanced expression between pro-death and pro-survival members of the apoptotic pathways, while in cancer cells over-expression of pro-survival members in conjunction with heightened barriers to cell death activation favors tumor establishment and growth. Key signaling pathways have been shown to lead to apoptosis. The intrinsic or mitochondrial pathway is characterized by the release of pro-apoptotic factors from mitochondria, including cytochrome c and Smac/DIABLO. Cytochrome-c binds to apoptotic protease-activating factor-1 (APAF-1), and leads to the activation of caspase-9 (2, 3). The extrinsic or death ligand pathway is triggered by ligands (e.g., TRAIL, TNF-α) binding to its corresponding receptor member of the TNF superfamily, which leads to the activation of intracellular caspase-8. In type I cells, processed caspase-8 directly activates other members of the caspase family, and triggers the execution of apoptosis of the cell. In type II cells, apoptosis signaling involves truncated BID to cross-activate the intrinsic pathway, thus allowing for mitochondrial amplification of apoptosis induction (4, 5). Regardless of the pathway of apoptosis activation or whether type I or II cells are involved, both intrinsic and extrinsic pathways ultimately converge to a final downstream pathway involving the activation of caspases-3, -6 and -7 that cleave PARP and other substrates, ultimately culminating in apoptotic cell death (6). The inhibitors of apoptosis (IAP) proteins are characterized by the presence of at least one baculovirus IAP repeat (BIR) structural domain (7–9). Among the IAP members, XIAP (X-linked IAP, BIRC4) is a critical barrier to apoptosis induction because of its robust affinity to bind and inhibit initiator caspase-9 and effector caspases -3 and -7 (10–12), thus effectively functioning to prevent cell death activation and sustaining survival of the cancer cell.

MicroRNAs are 22–23 nucleotide long noncoding RNAs that exert repressive effects on translation by targeting the 3’ UTR via a 6–8 nucleotide seed region that is critical for the coordination of the miRNA-mRNA complex (13). Recent reports demonstrate that microRNAs regulate IAP expression in a variety of cell types. Among the IAP members, survivin has been shown to be targeted by miR-494 and miR-320a in TEL-AML1+ leukemias (14); by miR-218 in nasopharyngeal carcinoma (15); by miR-708 in renal cancer cells (16); and by miR-203 in prostate cancer cells (17). As of yet, no microRNAs have been identified for c-IAP1 and c-IAP2. For XIAP, reports demonstrate that miR-23a or miR-200bc/429 are associated in altered conditions such as cerebral ischemia (18) or chemotherapy resistance in highly selected cancer cell clones (19), respectively. What relationship exists between microRNAs and XIAP in the basal state of cancer cells remains unreported. Thus, we hypothesize that the XIAP 3′ UTR, spanning approximately 6kb, can potentially harbor binding sites for microRNA participation in XIAP translational regulation.
In this study, we sought to identify microRNAs that specifically target the uncharacteristically long XIAP 3′UTR and consequently regulate the expression levels of XIAP. We employed a heuristic algorithm incorporating bioinformatics analysis with in vitro screening of cancer cells to identify microRNA candidates with the potential to interact with XIAP mRNA. Accordingly, we demonstrate a specific binding of miR-24 to the XIAP 3′ UTR that robustly represses XIAP translation, and establish a novel role for miR-24 and XIAP in the modulation of the apoptosis threshold in cancer cells. In addition, a microRNA cluster anchored by miR-24 is identified. As miR-24 has been reported to target several other mRNAs not directly involved in apoptosis (20–24), we also examined whether these alternative mechanisms were active in our cells. To the best of our knowledge, this is the first report demonstrating a novel role for miR-24 in determining apoptotic cell death resistance in cancer cells, and these data provide additional elucidation of microRNA regulation of the apoptosis pathway.

Results

Identification of microRNA candidates targeting XIAP 3′UTR

Using a heuristic algorithm incorporating computational predictive analysis and in vitro screening, a refined set of microRNA candidates targeting the XIAP 3′UTR was derived (Figure S1). The DIANA-microT v3.0, TargetScans, and miRanda databases identified 51 initial candidate miRNAs with the potential to interact with the XIAP 3′UTR from the total pool of human microRNAs in the Sanger database (Table S1). The candidate list was further reduced by analyzing the structural symmetry of predicted microRNA-mRNA binding complexes and their respective predicted Gibbs free-energy values, arriving at 17 candidate microRNAs (Table S2). Using primers specific for each microRNA candidate and a real-time RT-PCR based platform, we assayed the presence and relative abundance of each of the computationally-identified candidate miRNAs in lung cancer cell lines (CALU-1, A549, H1437, H292), cervical carcinoma (HeLa), esophageal cancer cell line (OE21), breast cancer cells (SK-BR-3, MDA-MB-468), and normal small airway epithelial cells (SAEC) (Figure S1). From the 17 miRNAs, miRNA-24 was the only miRNA which exhibited an inverse correlation with XIAP protein levels (Figure 1A & B). Consistent with microRNA’s expected repressive function as a translational inhibitor, miR-24 was well expressed in CALU-1, H292, and SAEC--cells with lower XIAP protein expression. In contrast, lower levels of miR-24 were found in H1437, HeLa, OE21, SK-BR-3, MDA-MB-468, and A549 cells and correlated with relatively higher XIAP expression.

Genetic basis of miR-24 expression

Per previous reports, miR-24 transcripts can originate either from chromosome 9 or 19 (25, 26). Also, the UCSC genome database predicts a set of microRNAs in close proximity to the miR-24 locus on each of these two chromosomes. On chromosome 19, miR-24-2, miR-23a, and miR-27a form one microRNA cluster (miR-24-2 cluster); while on chromosome 9, another miR cluster is formed by miR-24-1, miR-23b, and miR-27b (miR-24-1 cluster). To determine the chromosomal origins of the mature miR-24 seen in cancer and normal cells, the precursors of mature miR-24 were measured using primers specific to either the primary microRNA (pri-miR) cluster of miR-24-2 or miR-24-1. The expression profile of the
nuclear-derived primary microRNAs arising from chromosome 19 exhibits a perfect pattern of concordance to the levels of mature miR-24 expression (Figures 1A and 1C-E) while the pri-miR cluster from chromosome 9 does not (Figure S2), suggesting chromosome 19 to be the origin of mature miR-24 in these cells.

In order to identify whether structural chromosomal alterations may account for the expression levels of miR-24 in cancer cells, array-CGH was performed on genomic DNA extracted from H1437, A549, and CALU-1 cells. At the 19p13.13 locus, which corresponds to the miR-24-2 coding region, H1437 and A549 cells exhibit negative Log2 ratios (-0.193 and -0.578, respectively), indicating significant genomic loss in the miR-24 coding region in these two cancer cells compared to the normal human chromosome 19 (Figure 2A). In our CALU-1 data and those previously reported of H292 cells (27), the region of chromosome 19 spanning the miR-24-2 cluster is intact and thus correlates with a normal expression of mature miR-24 expression and normal levels (low) of XIAP (Figure 2A). Spectral karyotype analysis (SKY) of these same cells confirms that the relative loss of chromosome 19 in H1437 is mediated by monosomy 19 (Figure 2B); and in A549 cells, one normal chromosome 19 is present along with another chromosome 19 exhibiting a partial p-arm deletion (Figure 2B), thus accounting for an overall gene copy number reduction in cells which express markedly attenuated levels of mature miR-24 and high XIAP protein levels (complete SKY and aCGH data shown in Figure S3). In published data of HeLa cells analyzed with SKY (28), a relative reduction in gene copy number at the miR-24 locus is also seen and therefore accounts for a low basal miR-24 expression in these cancer cells as well.

As gene silencing can also occur via an epigenetic mechanism, the putative miR-24 promoter region (29), was analyzed for the presence of CpG islands that can serve as targets for methylation-mediated silencing. While no formal CpG islands are found in this promoter region, 13 CpG base-pair sites can be identified along this span of upstream DNA (Figure S4A). Next, the genomic DNA of lung cancer cells and normal lung cells were subjected to bisulfate modification and subsequent sequencing of the promoter region was performed in order to uncover DNA methylation at the CpG sites. Although the majority of CpG sites are unmethylated, some methylated CpG sites were identified (Figure S4A). CALU-1 cells, which had the greatest number of methylated CpG sites in the promoter region, were subjected to demethylation treatment with 5-aza-2′-deoxycytidine (DAC) in increasing concentrations; however, no increase in mature miR-24 levels resulted despite an appropriate rise of NY-ESO-1 (Figure S4B), a cancer-testis antigen normally under methylation-mediated silencing (30, 31). Additionally, treatment of CALU-1 cancer cells with the histone deacetylase inhibitor trichostatin A (TSA), either alone or in combination with DAC, also did not alter mature miR-24 levels (Figure S4B), thus suggesting that DNA promoter methylation does not contribute a significant regulatory role in the expression of miR-24.

**XIAP 3′UTR is critical to miR-24’s actions**

To evaluate whether the XIAP 3′UTR is necessary for the effects of miR-24, a stably-transfected cancer cell line (A549/XIAP-GFP) expressing the XIAP coding region without
its 3′UTR was generated. When treated with exogenous miR-24, the level of endogenous XIAP decreases but the XIAP-GFP fusion protein level is not attenuated because it lacks a 3′UTR (Figure 3A). Given that XIAP is a major barrier to apoptosis induction, down-regulation of XIAP is expected to restore apoptosis potential. In empty vector control cells (A549/GFP-empty vector), treatment with miR-24 reduces endogenous levels of XIAP and resulted in increased sensitivity to TRAIL-induced cytotoxicity (Figure 3B). In contrast, the expression of miR-24-insensitive XIAP-GFP fusion protein in A549/XIAP-GFP cells functions to significantly abrogate apoptosis from miR-24 and TRAIL combination treatment (Figure 3B). Thus, miR-24 sensitization of TRAIL-induced apoptosis is largely mediated through down-regulation of endogenous XIAP expression and the presence of the XIAP 3′UTR is critical for the effects of miR-24.

Identification & confirmation of miR-24 target site in XIAP 3′UTR

To further explore the nature of interaction between miR-24 and the XIAP 3′UTR, three putative binding sites for miR-24 in the XIAP 3′UTR were identified by computational analysis. To verify miR-24 direct binding, three fragments of the XIAP 3′UTR, each containing a single putative miR-24 binding site, were cloned and inserted into separate luciferase reporters (XIAP Luc-A, XIAP Luc-B, and XIAP Luc-C, respectively) and transfected into HEK293 cells (Figure 4A–D). Treatment of transfected HEK293 cells with miR-24 markedly reduced luciferase activity only in cells with XIAP Luc-A, the construct containing putative binding site-A (nucleotides 2294-2310), and there was no significant reduction in luciferase activity by miR-24 in XIAP Luc-B or XIAP Luc-C cells (Figure 4B), suggesting that site A is a true binding site of miR-24. Correspondingly, the addition of anti-miR-24 to XIAP Luc-A resulted in an increase in luciferase activity above control baseline levels (Figure 4C). To further demonstrate the specific nature of the miR-24-mRNA interaction, the sequence within the XIAP Luc-A site binding to the seed region of miR-24 was mutated, resulting in loss of miR-24 mediated repression of luciferase activity (Figure 4D). These data support that miR-24 directly targets the 3′UTR of XIAP mRNA at nucleotides 2294-2310 (site A) to exert an inhibitory effect on XIAP protein expression and that this site is a major miR-24 binding site.

Functional significance of miR-24

XIAP is a critical barrier to apoptotic cell death because it can directly inhibit the activation of caspases and therefore block normal apoptosis activation (7–9). Accordingly, the functional significance of miR-24 was further evaluated in conditions of miR-24 over-expression and attenuation in multiple cancer cells. Transfection of miR-24 precursors in A549 cells resulted in a robust 23-fold increase in mature miR-24 levels (Figure 5A) and a significant reduction in XIAP protein levels compared to mock transfection with negative control microRNA (Figure 5B). As in A549 cells, miR-24 over-expression in OE21 esophageal or HeLa cervical cancer cells also significantly reduces XIAP protein levels (Figure 5B). Unlike siRNA directed against XIAP, miR-24 mediated reduction of XIAP protein levels is not mediated by mRNA degradation (Figure 5C), and instead is consistent with a mechanism of action via translational repression. Conversely, when endogenous miR-24 levels are decreased through exogenously provided miR-24-specific anti-microRNAs (anti-miR-24), there is a reduction in endogenous miR-24 levels and a
corresponding increase in endogenous XIAP protein expression (Figures 5D and 5E). Similarly, treatment of normal SAEC cells with anti-miR-24 also results in increased endogenous XIAP protein levels and in resistance against apoptosis as well (Figures 5F and 5G). Next, a broad range of cancer cell lines that are highly resistant to TRAIL activation of apoptosis were treated with combination miR-24 precursors and soluble recombinant human TRAIL. Sensitivity to TRAIL-induced cytotoxicity was restored in A549, H292, OE21, HeLa, SK-BR-3, and MDA-MB-468 (Figure 6A). In combined miR-24 over-expression and TRAIL, caspase-3 activity became markedly elevated in A549 and H292 cells, as detected by fluorometric caspase function assay (Figure 6B), and a concomitant cleavage of full length caspase-3 into its activated form is observed (Figure 6C). As controls, neither TRAIL treatment nor miR-24 over-expression alone induces caspase-3 activation. Furthermore, pre-treatment with the pan-caspase inhibitor z-VAD-FMK inhibited the caspase-3 activity, caspase cleavage, and decreased cell viability seen in both A549 and H292 cells due to miR-24 and TRAIL co-treatment (Figures 6B, 6C, and 6D). Another hallmark of downstream apoptosis activation, cleavage of poly (ADP-ribose) polymerase (PARP), which is involved in repair of DNA strand breaks and cleaved during apoptosis by caspase-3 and caspase-7, also resulted following miR-24 over-expression and TRAIL treatment but is absent when pan-caspase inhibition is enforced (Figure 6C). These data indicate that miR-24 over-expression in cancer cells can facilitate TRAIL-induced apoptosis via restored function of the canonical apoptosis pathway.

**Discussion**

In this study, we demonstrated that miR-24 directly down-regulates XIAP protein expression, a critical barrier to apoptosis induction, by targeting the 3′UTR of the XIAP mRNA. In cancer cells, observed miR-24 levels correlated inversely with XIAP protein expression, and low basal expression of miR-24 is attributable to genomic DNA loss at the miR-24 gene locus. Our results also illustrated that over-expression of miR-24 attenuated XIAP levels to sensitize multiple intrinsically apoptosis-resistant cancer cells to cell death by TRAIL treatment.

The expression levels of XIAP can vary between cells of different cancer cell histology and sub-types; however, the mechanisms that regulate XIAP expression are not yet fully understood. XIAP gene variations are uncommon and the most frequent SNPs of XIAP do not correlate with cancer presentation (32, 33). As such, aberrations of the XIAP gene do not explain the diverse range of XIAP expression observed in primary tumors and cancer cell lines. Another possible level of regulation can occur at translational initiation, involving the 5′UTR of the XIAP mRNA, which may contain a 162-nucleotide long internal ribosome entry site (IRES) motif that enables cap-independent translation in conditions of cellular stress (34). As a consequence, a long isoform of the XIAP mRNA (containing IRES) and a shorter isoform (without IRES) has been described, with the short isoform expressed under basal conditions (35). The XIAP down-regulated by miR-24 in multiple cancer cells is likely to be translated predominantly from the short XIAP mRNA isoform and not the IRES-driven XIAP mRNA variant. In both reported XIAP isoforms, the 3′UTR appears to be unaltered. Here, we show that miR-24 can regulate XIAP protein expression by direct targeting of the 3′UTR without alteration of XIAP mRNA levels, thus implying that translational repression
for XIAP must take place at the initiation or post-initiation level. Currently, how miRNAs specifically mediate repression of protein translation remains poorly defined (36). Previous reports have shown that the miRNA-loaded RISC complex can inhibit translational initiation through targeting of the cap-binding protein eIF4E (37). However, in select cases of messenger RNAs with IRES, translation is unperturbed by miRNA (37–39). In contrast, it has also been shown that translation initiated by the cap-independent IRES of hepatitis C virus (HCV) and cricket paralysis virus (CrPV) are repressible by miRNAs (40). Additional translational inhibition can also occur at the post-initiation level as the miR-RISC complex can promote ribosome dissociation from mRNAs (40) or stimulate deadenylation of the mRNA poly A tail (41). Hence, how microRNAs manifest translational repression remains controversial.

In the apoptosis pathway, there is accumulating experimental data which implicate microRNAs as important determinants of key member expression. MicroRNAs (such as miR-15/16, miR-195, miR-181b, miR-34a) have been shown to modulate the expression of bcl-2 in chronic lymphocytic leukemia (CLL), colorectal cancers, multidrug-resistant human lung cancer cells, human gastric cancer, and Alzheimer’s disease (42–45), respectively. And in human embryonic kidney cells, miR-27a attenuates the expression of Fas associated protein with death domain (FADD) (46). With respect to the IAPs, multiple microRNAs (miR-542-3p, miR-494, miR-320a, miR-218, miR-708, and miR-203) have been found to modulate survivin expression (14–17, 47). In contrast, no miRNAs have been identified for cIAP-1 and cIAP-2 to date; however, we speculate that it is likely that microRNAs also exist which participate in regulating the expression of these IAPs as well. Meanwhile, survivin’s role as a true IAP has been questioned by some based on major differences in structural features and its caspase inhibition profile (48) compared to the other IAPs. Thus, we speculate that survivin’s multiple miRNA relationships are possibly related to functions of survivin other than in apoptosis regulation.

As for XIAP, the data presented here reveals the role of direct microRNA control of basal XIAP expression in cancer and normal cells, whereas other microRNA reports regarding XIAP have shown a miR-XIAP relationship in specialized conditions. Interestingly, miR-23a, which has been reported to target XIAP in gender based conditions of cerebral ischemia (18), is also a member of the miR-24-2 microRNA cluster presented here. While we found that miR-23a can also attenuate XIAP in cancer cells in vitro, it did so to a lesser degree than mature miR-24 (Figure S5A). Further, miR-23a and TRAIL was unable to functionally re-activate apoptosis as compared to miR-24 with TRAIL (Figure S5B). Why miR-24 appears to take a leading role in XIAP regulation over other miRNA members in its cluster is unclear. XIAP regulation by microRNA was also found in rheumatoid arthritis synovial fibroblast cells in a recent report, contemporary with the work presented here, which demonstrated that the passenger strand of miR-34a* was found to target the XIAP 3’UTR to attenuate XIAP levels, while the corresponding mature miR-34a strand appeared to be inactive (49). In aggregate, our data along with those of others re-affirm that microRNAs participate in direct regulation of core pathways involved in determining cancer cell death versus cell survival. Moreover, aberrant microRNA expression may be integral mediators of carcinogenesis and cancer therapy resistance.
Some have proposed that a single miRNA can repress the expression of multiple proteins and modulate a wide variety of biological processes (50, 51); although how one miRNA coordinates its effects on multiple targets is not well understood. miR-24 has been reported to decrease human dihydrofolate reductase (DHFR) to inhibit cancer cell proliferation, but the effects of a naturally occurring SNP (C829T) near the miR-24 binding site in the DHFR gene can also result in DHFR overproduction and methotrexate resistance in several malignancies (20). miR-24 has also been reported to inhibit erythropoiesis by targeting activin type I receptor ALK4 (21) and suppress the expression of p16 in human diploid fibroblasts and cervical carcinoma cells (22). Others have observed the down-regulation of histone variant protein H2A.X is mediated by miR-24, rendering terminally differentiated blood cells vulnerable to DNA damage (23). In addition, miR-24 may regulate cell-cycle progression by suppressing the expression of E2F2 and MYC (24). More recently, it has been reported that miR-24 can repress cellular apoptosis by targeting caspase-9 and apaf-1 in the developing neural retina in Xenopus and Fas-associated factor-1 in human cervical and gastric cancers, respectively (52, 53). However, the down-regulation of caspase-9 and apaf-1 would theoretically raise the apoptosis threshold and therefore retard apoptosis, which is contrary to our experimental data. In our experience, the levels of DHFR, ALK4, p16, c-Myc, H2A.X, E2F2, Apaf-1, and caspase-9 were not consistently altered across cell lines after miR-24 administration (Figure S6). Thus, our data and those of others support miR-24 as an active participant in the regulation of several cell-specific targets but the expression of these different biological functions appears to be executed in a cell-type and perhaps species-dependent manner.

The loss of microRNA expression is a common feature of cancer (54). Our genomic analyses revealed that the decreased expression of miR-24 in cancer cells such as A549 and H1437 is consistent with overall copy number reduction due to the loss of chromosome band 19p13.13, which spans the miR-24 cluster ORF. Alternatively, epigenetic silencing has also been reported to regulate specific microRNAs (55). Upstream of the miR-24 coding region, no classically-defined CpG islands are present that would accommodate classic silencing through CpG-methylation. In our experiments, the methylated CPG sites found in the miR-24 promoter appear inactive, and direct treatment with epigenetic modifying agents, such as DAC and TSA, did not consequently alter the miR-24 levels in cancer cells. Thus, we conclude from our data that DNA promoter methylation does not appear to play a role in miR-24 expression and chromosomal alteration is a main determinant.

We also demonstrated here that miR-24 can modulate XIAP expression to permit normalized apoptosis induction in inherently TRAIL-resistant cancer cells, and that the resulting apoptosis is caspase-mediated. Similar to siRNA, the actions of miR-24 on XIAP are specific and does not target or alter other apoptosis-related proteins, including cIAP-1, cIAP-2 survivin, Bcl-2, Bcl-xL, cFLIP, and Mcl-1 (Figure S7). The over-expression of miR-24 can significantly abrogate the effects of XIAP on apoptosis, indicating the important role that XIAP and the loss of miR-24 play as key barriers to apoptosis in cancer cells. XIAP is known to inhibit the activation of caspases-3, -7 and -9, thereby blocking both the intrinsic and extrinsic apoptotic pathways. As over-expression of XIAP contributes to the resistance to apoptosis-inducing treatments, attenuation of XIAP via miR-24 augmentation could be an important future molecular approach for modulating apoptosis.
In summary, this study shows for the first time that miR-24 directly modulates the expression of XIAP, and genomic copy number loss of the miR-24 cluster is consistent with loss of endogenous miR-24 in cancer cells. Moreover, re-establishment of miR-24 expression in cancer cells can lead to restoration of functional apoptosis. Collectively, these findings also suggest that augmentation of native miR-24 levels in cancer tumors over-expressing XIAP may be a possible alternate strategy for treating apoptosis-resistant human cancer.

Materials and methods

Cell lines and drug treatment

Human non-small cell lung cancer cell lines (A549, H1437, CALU-1, H292), breast cancer cell lines (SK-BR-3, MDA-MB-468), and the cervical carcinoma cell line HeLa were obtained from ATCC (Manassas, VA, USA). Esophageal squamous carcinoma OE21 cells were obtained from Sigma (Saint Louis, MO, USA). Normal human small airway epithelial cells (SAEC) were purchased from Lonza (Walkersville, MD, USA). A549 cells were cultured in Ham’s F12K medium (Gibco, Carlsbad, CA). CALU-1 and SK-BR-3 cells were cultured in McCoy’s 5A medium (Gibco). H1437, HeLa and OE21 cells were cultured in RPMI-1640 medium. MDA-MB-468 cells were grown in Leibovitz’s L-15 medium (Gibco). SAEC cells were maintained in small airway epithelial cell growth medium (Lonza). All cells were cultured at 37°C in a humidified atmosphere, with 5% CO2 in media supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA) and 1% penicillin/streptomycin (Gibco). Hydrogen peroxide (H2O2), 5-aza-2′-deoxycytidine (DAC) and trichostatin A (TSA) were purchased from Sigma Chemical (St. Louis, MO, USA). The effects of DAC and TSA treatment on gene expression were determined after exposure cells to 0.1–3 μM of DAC for 72 hours followed by normal media or 300 nM TSA for 24 hours or the combination. Caspase inhibitor z-VAD-FMK was purchased from R&D System (Minneapolis, MN). The effects of z-VAD-FMK and TRAIL on caspase-3 activity were determined after pretreatment of cells with 40 μM of z-VAD-FMK for 30 minutes followed by 100 ng/mL TRAIL for 4 hours. Correlative cell viability assays were performed with a 1 hour pre-treatment with 40 μM of z-VAD-FMK followed by 100 ng/mL TRAIL for 24 hours. To establish A549/XIAP-GFP clones, A549 cells were transduced with lentiviral p-Receiver-lv19-XIAP (XIAP-GFP) or p-Receiver-lv19 empty vector (GFP-empty vector) (GeneCopoeia, Rockville, MD). Serial dilutions of the cells were cultured in media containing neomycin (500 ng/ml), and colonies were propagated in this medium for isolation of individual cell lines.

microRNA and siRNA transfection

For microRNA and siRNA studies, cells were plated in 6 or 12 well plates 1 day prior to transient transfection. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in OPTI-MEM media (Gibco) and microRNA precursor miR-24 (40 nM), pre-miR precursor negative control (40 nM), anti-miR microRNA inhibitor miR-24 (40 nM), or XIAP siRNA 5′-CAUGCACUGUAGAUGAUGGCAAU-3′ (40 nM) (Invitrogen). Analyses were performed 48–72 hours after transfection.
Heuristic Computational Analysis of microRNA candidates

DIANA-microT v3.0 (56), TargetScans v4.2 (57), miRanda (58) and RNAhybrid (59) software were used to identify potential microRNA candidates.

RNA isolation and quantitative real time polymerase chain reaction (qRT-PCR)

RNAs were isolated with the mirVana miRNA Isolation Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s instruction for total RNA isolation. Expression of selected miRNA was analyzed by qRT-PCR using TaqMan MicroRNA Expression Assays (Applied Biosystems). The mature miRNA expression levels were normalized to RNU6B (Applied Biosystems). The level of XIAP mRNA was quantified by TaqMan gene Expression Assays (Hs00236913_m1, Applied Biosystems) according to the manufacturer instructions. Expression levels of the genes were normalized to 18S rRNA (Hs99999901_s1, Applied Biosystems). The transcription of primary microRNA (pri-miRNA) was quantified by TaqMan Pri-miR assays (Hs03303056_pri, Hs03294931_pri, Applied Biosystems). The high capacity cDNA reverse transcription kit (Applied Biosystems) was used to convert RNA to cDNA. The primary miRNA expression levels were normalized to GAPDH (Hs99999905_m1, Applied Biosystems).

Cloning and mutagenesis 3′UTR seed regions

To generate a luciferase reporter to evaluate miRNA activity, 3 fragments of XIAP 3′UTR (2214-2834, 3294-4008 and 6061-7558) were amplified by RT-PCR from total RNA isolated from H460 cells and inserted into pMIR-reporter vector (Applied Biosystems) at SpeI/MluI restriction site downstream of the firefly luciferase open reading frame. Each fragment contains one of the 3 putative miR-24 binding sites. The QuikChange XL site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA) was used to generate the mutation in the seed region binding sites. Three nucleotides (nt 2305-2307) in the 3′UTR of XIAP gene were deleted by PCR from the WT XIAP. Wild type and mutant inserts were confirmed by sequencing. The primers used for cloning are as follows:

| Binding site | Forward | Reverse |
|--------------|---------|---------|
| A            | 5′-GGACTAGTAGAATACTATCGAGCCAACATGTACTG-3′ | 5′-TTACGCCCTACTAGCAAGGATTAAGGATGAATCT-3′ |
| B            | 5′-GGACTAGTAACCTATTGTAGAGGTGAGTAAGGCAT-3′ | 5′-TAACGCGTCACAACTAGATTGAACTACCTACCTGAC-3′ |
| C            | 5′-GGACTAGTAGTGAGCTATGATTGTGCCACTGTAC-3′ | 5′-ATACGCGTTGAGGACGCTTAAGTCCCTTAGATAA-3′ |

Western blotting and Caspase activity assay

Cells were lysed using RIPA lysis buffer (Millipore Corp., Billerica, MA, USA) with protease inhibitor cocktail tablet (Upstate, VA, USA). Cell lysates, containing equal amounts of protein, were mixed with SDS protein gel loading solution, resolved in SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked in 5% non-fat milk then incubated with the appropriate primary antibodies (1:1000): anti-XIAP (BD Biosciences, San Diego, CA, USA), anti-DHFR (BD Biosciences), anti-Caspase-3 and
anti-Caspase-9 (Cell Signaling Technology, Danvers, MA, USA), anti-Apaf-1 (Cell Signaling), anti-PARP (Cell Signaling), anti-survivin (Cell Signaling), anti-p16 (Cell Signaling), anti-c-Myc (Cell Signaling), anti-cIAP-1 and anti-cIAP-2 (R&D Systems), anti-ALK4 (R&D systems), anti-H2A.X (Millipore), anti-E2F2 (Santa Cruz Biotechnology, CA, USA), anti-Bcl-2 (Santa Cruz Biotechnology), anti-Bcl-xL (Epitomics, Inc., CA, USA), anti-cFLIP (Calbiochem), anti-Mcl-1 (Cell Signaling). Membranes were then incubated in either goat anti-rabbit or anti-mouse, or rabbit anti-goat secondary antibodies (1:5000), exposed to the Supersignal West Femto Substrate (Pierce, Rockford, IL, USA) and visualized under the UVP imager (UVP, Upland, CA). For detection of internal control (GAPDH), the membrane was stripped in stripping buffer for 30 min at 37°C and re-probed with anti-GAPDH antibody (BD Biosciences). Activated caspase-3 levels were measured using the Caspase-3 Fluorometric Assay kit (R & D Systems).

Cell viability assay
A549, OE21, HeLa, H292, SK-BR-3, and MDA-MB-468 cells were seeded at 1–2 × 10^5 cells per well in 12-well plates. Then, cells were transfected with pre-miR™ miRNA precursor (hsa-miR-24 or negative control #2) for 48 hours and incubated with different concentrations of TRAIL for additional 48 hours. MTT (Sigma) was added to each well at a final concentration 0.5 mg/ml and incubated for 3 hours. MTT was removed and cells were incubated overnight in mineral oil (Fisher). The formation of colored formazan dye was assessed colorimetrically at 550 nm. Results are expressed as percentage loss of cell viability compared with control.

Metaphase harvesting and SKY analysis
Metaphase chromosomes for SKY were prepared after exposing the tumor cells (passages 2–6) to colcemid (Roche, Indianapolis, IN, USA) for 1–3 hours with a final concentration 0.1 μg/ml. The cells were lysed in hypotonic solution (0.075 M KCl), and the nuclei were fixed in methanol and acetic acid (3:1). SKY was performed for the identification of chromosomal abnormalities according to the technique and protocol previously used (60). Differentially labeled chromosome-specific painting probes were hybridized simultaneously onto metaphase chromosomes. A minimum of 10 SKY and DAPI metaphase images were acquired and analyzed for each cell line. The karyotype findings were described in accordance with the ISCN nomenclature rules (ISCN, 2005).

DNA extraction and Array CGH
DNA was extracted using standard methods. Oligonucleotide-based array CGH was performed according to the protocol provided by the manufacturer (Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis, Agilent Technologies, Santa Clara, CA, USA), with minor modifications. Commercially available pooled control DNA (Promega) was used as sex-matched reference DNA in the cancer cell line hybridizations. Briefly, 3 μg of genomic DNA was digested for 2 hours with AluI and RsaI (Promega) and purified using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). Sample and reference DNA was labeled for 2 hours with Cy3-dUTP and Cy5-dUTP (Promega), respectively, in a random priming reaction using Bioprime Array CGH Genomic Labeling Module (Invitrogen). Unincorporated nucleotides were removed using Microcon YM-30 columns.
Cy3 and Cy5-labeled samples were combined in equal amounts according to the incorporation of labeled nucleotides measured with the Nanodrop. A 244K oligonucleotide-based human genome microarray (Agilent Technologies) was subjected to hybridization for 40 hours at 65°C. The microarrays were then washed using the manufacturers' recommended conditions, and were scanned using a laser scanner (Agilent Technologies). Agilent Feature Extraction™ software (version 9.2, Agilent Technologies) was applied for image analysis. Agilent CGH Analytics 4.0 software (Agilent Technologies) and Nexus Copy Number 4.0 (BioDiscovery, Inc) were used to visualize and analyze the data. Circular binary segmentation algorithm interpreted in Agilent CGH Analytics was applied to identify copy number changes.

**Statistical analysis**

Triplicate experiments represent data pooled from three independent experiments. Statistical evaluation for data analysis was determined by using the paired Student’s t-test and ANOVA with post hoc testing for comparison of multiple groups. All data were shown as the mean ± standard deviation. A statistical difference of p<0.05 was considered significant.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Down-regulation of mature miR-24 correlates with increased XIAP protein levels. (A) Real-time RT-PCR analysis of mature miR-24 expression in normal (SAEC), NSCLC (CALU-1, A549, H1437, H292), cervical carcinoma (HeLa), esophageal (OE21) and breast (SK-BR-3, MDA-MB-468) cancer cells. Bars represent mean and standard deviation from triplicate experiments. (B) Representative western blot of XIAP protein in normal and cancer cells. Quantitative XIAP protein levels are normalized to SAEC. GAPDH served as a loading control. (C–E) Real-time RT-PCR quantification of the primary (Pri-miR) miR-24-2 cluster located on chromosome 19 (C), which also contains mature miR-23a (D) and mature miR-27a (E). Bars represent mean and standard deviation from triplicate experiments.
Figure 2.
Chromosomal loss in the miR-24 coding region of A549 and H1437. (A) Summary of array-CGH data from chromosome 19 in H1437, A549, and CALU-1 NSCLC cells. The miR-24 coding region is located at 19p13.13. Genomic loss is represented by bars to the left of center or negative Log2 ratios while genomic gain is represented by bars to the right of center or positive Log2 ratios. (B) Representative spectral karyotyping (SKY) analysis of chromosome 19 in A549 (upper) and H1437 (lower) lung cancer cell lines. A549 cells carry a partial p-arm deletion in chromosome 19. H1437 has monosomy of chromosome 19 in over 40% of cells analyzed.
Figure 3. Over-expression of miR-24 targets endogenous but not ectopic XIAP in A549/XIAP-GFP stably transfected cells. (A, B) Stable cell line A549/GFP-empty vector or A549/XIAP-GFP without the 3’ UTR of XIAP was transfected with pre-miR-24 or miR negative control for 48 hours, and then incubated with 100 ng/ml of TRAIL for additional 48 hours. (A) Representative western blot of endogenous and ectopic XIAP protein levels. miR-24 over-expression reduces endogenous XIAP protein levels but has no effect on ectopic XIAP protein levels. (B) MTT cell viability assay. Ectopic expression of the XIAP-GFP fusion protein inhibits the effect of pre-miR-24 and TRAIL treatment on cell viability. Bars represent cell viability as a percentage relative to untreated cells and standard deviation from triplicate experiments (***p<0.05).
Figure 4.
miR-24 binds with specificity to the 3′ UTR of XIAP mRNA. (A) The 3′ UTR of XIAP mRNA harbors 3 putative miR-24 binding sites -A, -B, -C. Schematic representation of firefly luciferase reporter constructs (XIAP Luc-A, Luc-B, and Luc-C, respectively) for truncated XIAP 3′ UTR miR-24 target sites. (B, C) HEK293 cells transfected with the luciferase reporter constructs and pre-miR miRNA-24 precursor (pre-miR-24) or miR negative control. Over-expression of miR-24 by pre-miR-24 significantly decreases the luciferase activity of XIAP Luc-A (B) while inhibition of miR-24 via anti-miR-24 results in a significant increase in luciferase activity (C). (D) Comparison of luciferase activity of wild type (WT) and mutant (MT) XIAP Luc-A constructs following transfection with pre-miR-24 or negative control in HEK293. XIAP Luc-A/ MT significantly inhibits the effects of miR-24 over-expression on luciferase activity. Luciferase assay was performed 24 hours after transfection. The data are normalized to β-galactosidase activity. Values are shown as the percent of luciferase expression compared with the control from triplicate experiments (***p<0.01).
Figure 5. miR-24 modulates XIAP expression. (A) Transfection of pre-miR-24 in A549 cells results in a 23-fold increase in miR-24 expression compared to miR negative control. Bars represent miR-24 expression relative to control and standard deviation from triplicate experiments (**p<0.01). (B, C) Representative western blots of XIAP protein levels (B) and expression of XIAP mRNA (C) following transfection with pre-miR-24 or XIAP siRNA. Overexpression of miR-24 results in a decrease in XIAP protein levels but does not alter XIAP gene expression. (D, E) Reduction of miR-24 expression by anti-miR-24 inhibitor (D) increases XIAP protein levels (E). Quantitative protein levels and mRNA expression are normalized to untreated from triplicate experiments (**p<0.01). (F, G) Anti-miR-24...
inhibitor increased XIAP expression in SAEC cells (F) and protects SAEC cells from hydrogen peroxide-induced apoptosis (G).
Figure 6. miR-24-mediated XIAP suppression restores TRAIL-induced apoptosis in TRAIL-resistant cells. (A) Cell viability of A549 (i), H292 (ii) OE21 (iii), HeLa (iv), SK-BR-3 (v), and MDA-MB-468 (vi) cells following a 48-hour transfection with pre-miR-24 or miR-negative control and an additional 48h treatment with increasing concentrations of soluble TRAIL (*p<0.05, **p<0.01). (B, C) miR-24 over-expression and TRAIL treatment increased caspase-3 activity in A549 and H292 cells while z-VAD-FMK inhibited TRAIL-induced caspase-3 activity in miR-24 over-expressed cells. Values represent caspase-3 activity relative to negative control from triplicate experiments as measured by fluorometric assay (**p<0.01). (C, D) Corresponding representative western blot of caspase-3 and PARP.
cleavage (C) and cell viability (D) following miR-24 over-expression and TRAIL treatment, with and without z-VAD-FMK pre-treatment.