AMACR Amplification in Myxofibrosarcomas: A Mechanism of Overexpression That Promotes Cell Proliferation with Therapeutic Relevance

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Abstract

Purpose: Myxofibrosarcomas frequently display arm-level gains on 5p. We characterized the pathogenic and therapeutic relevance of the γ-methylacyl coenzyme A racemase (AMACR) at 5p13.3.

Experimental Design: AMACR mRNA expression in myxofibrosarcomas was analyzed using the public transcriptome and laser-microdissected sarcoma cells. We performed florescence in situ hybridization (FISH) and immunohistochemistry in independent samples for clinical correlates. In AMACR-overexpressing myxofibrosarcoma cells and xenografts, we elucidated the biologic function of AMACR using RNA interference and explored the therapeutic effect and mechanism of an AMACR inhibitor, ebselen oxide.

Results: AMACR protein overexpression and gene amplification were significantly associated with each other (P < 0.001), with higher tumor grades (both P < 0.002), and univariately with worse metastasis-free survival (MFS; both P < 0.0001) and disease-specific survival (DSS; P = 0.0002 for overexpression; P = 0.00062 for amplification). AMACR protein overexpression also independently portended adverse outcome (DSS, P = 0.007; MFS, P = 0.001). However, 39% of AMACR-overexpression cases did not show gene amplification, implying alternative regulatory mechanisms. In myxofibrosarcoma cell lines, stable AMACR knockdown suppressed cell proliferation, anchorage-independent growth, and expression of cyclin D1 and cyclin T2. These growth-promoting attributes of AMACR were corroborated in the AMACR-silenced xenograft model and AMACR-underexpressed myxofibrosarcomas, showing decreased labeling for cyclin D1, cyclin T2, and Ki-67. Compared with fibroblasts, AMACR-expressing myxofibrosarcoma cells were more susceptible to ebselen oxide, which not only decreased viable cells, promoted proteasome-mediated degradation of AMACR protein, and induced cellular apoptosis in vitro, but also dose-dependently suppressed xenografted tumor growth in vivo.

Conclusions: Overexpressed AMACR in myxofibrosarcomas can be amplification-driven, associated with tumor aggressiveness, and may be relevant as a druggable target. Clin Cancer Res; 20(23); 6141–52. ©2014 AACR.

Introduction

Myxofibrosarcoma is a common adult sarcoma featuring multinodular growth of spindle to polygonal cells within variably myxoid stroma containing curvilinear vessels (1–3). It is characterized by distant metastases after relentless local recurrences and has an estimated 5-year overall survival rate of 75% (1–3). Local recurrent events have been reported to parallel the cytogenetic complexity (4). Given its wide histologic spectrum, from bland-looking lesions to pleomorphic sarcomas, myxofibrosarcoma presents a...
Translational Relevance

Encoding an enzyme regulating the β-oxidation of dietary branched-chain fatty acids, α-methylacyl coenzyme A racemase (AMACR) at 5p13.3 was differentially over-represented with upregulated mRNA expression in myxofibrosarcomas. AMACR overexpression can be driven by gene amplification in 20% of myxofibrosarcomas and associated with adverse outcome. In myxofibrosarcoma cell line and xenografted models, the tumor-promoting function of AMACR was linked to the heightened cell proliferation, concomitant with upregulated cyclin D1 and cyclin T2. In vitro and in vivo, AMACR-expressing myxofibrosarcomas were selectively susceptible to AMACR-inhibiting 3β-oxo-Δ7 sterol oxidase, which promoted proteasome-mediated degradation of AMACR protein and induced cellular apoptosis. Gene amplification can drive AMACR overexpression that exhibits pathogenetic and therapeutic relevance in myxofibrosarcomas.

Effects of AMACR overexpression that causes metabolic deregulation in cancer cells.

To our knowledge, this is the first study to characterize gene amplification as a mechanism that drives AMACR overexpression, with adverse prognostic implications in myxofibrosarcomas. In the AMACR-expressing cell lines and derived xenografts, we showed by RNA interference that the proliferation-promoting function of AMACR is linked to its induced expression of CCND1 and CCNT2. Furthermore, we demonstrated that myxofibrosarcoma cell lines and xenografts expressing AMACR were susceptible to a novel, nonsubstrate-based AMACR inhibitor, implicating AMACR as a druggable therapeutic target.

Materials and Methods

Analysis of published genomic profiling and transcriptomic datasets

We recently reported the array comparative genomic profiling data of our myxofibrosarcoma tissue and cell line samples (6), which are available in the Gene Expression Omnibus under accession name GSE35483. Nexus software (BioDiscovery) was used to profile the DNA copy number alterations across chromosome 5p and to depict a zoomed-in view of imbalanced genes, which were further screened for candidates displaying differential mRNA expression by reappraising published transcriptomic datasets of myxofibrosarcomas (GSE21122) versus non-neoplastic soft tissues. Those genes with concordant alterations in the genomic and expression profiling are considered more likely to contribute to myxofibrosarcoma pathogenesis. The raw CEL files obtained from Affymetrix U133A microarray platform were imported into Nexus Expression 3 software (BioDiscovery) to analyze all probe sets without preselection or filtering. Those genes with P < 0.001 were considered significant.

Tumor characteristics

Tissue procurement of myxofibrosarcomas was approved by the Institutional Review Boards of Chi-Mei (IRB09901-006) and Chang Gung (102-0670B) hospitals. The criteria for diagnosis and parameter assessment have been previously elaborated (5, 7, 16). We assembled 114 formalin-fixed primary myxofibrosarcoma samples from patients who received resection with curative intent, but not neoadjuvant radiation or chemotherapy, into recipient blocks of tissue microarrays (TMA) containing triplicate 1.0-mm tissue cores for each case. The locus-specific florescence in situ hybridization (FISH) targeting the AMACR gene and AMACR immunostaining were performed on TMA sections, yielding 105 cases informative for both data types (Table 1) and independent of fresh samples for prior genomic profiling and quantification of AMACR mRNA levels (6). Additional clinicopathologic factors are detailed in Supplementary Method S1.

FISH

The AMACR gene copy number in myxofibrosarcoma tissues was assessed on 4-μm TMA sections by the locus-specific FISH, for which a bacterial artificial chromosome
probe (CTD-2340N2; Invitrogen), spanning AMACR at 5p13.3, was labeled with spectrum orange. Given no DNA alterations in 19p12 in our prior genomic profiling data (6), we used a reference probe mapped to this region close to ZNF725 (CTB-28I9; Invitrogen) and labeled it with spectrum green. The average numbers of red and green signals were determined by examining approximately 200 tumor cells for each specimen. Gene amplification was defined as a ratio of the gene probe signal to the control probe signal exceeding two (17).

**Immunohistochemistry**

TMA sections were microwave-heated to retrieve tissue antigen, incubated with the primary antibodies against AMACR (1:350; Biocare Medical), cyclin D1 (1:50; Epitomics), cyclin T2 (1:100; Santa Cruz Biotechnology), Ki-67 (1:200; Abcam), and SKP2 (1:100; Zymed), and detected for protein expression using a ChemMate EnVision kit (Dako) as previously described (7, 15, 18). One pathologist (J. Lan) independently assessed immunohistochemical results to record mean percentages of labeling cells in the tumoral cytoplasm or nuclei for each case. Specifically, AMACR overexpression was defined as cases showing 50% or more tumor cells with moderate or strong cytoplasmic staining using the scoring method previously reported (15).

**Cell culture, RNA interference, and stable clones**

We previously reported the derivation and maintenance of OH931, NMFH-1, and NMFH-2 myxofibrosarcoma cell lines and CCD966SK dermal fibroblasts, the authentication of all cell lines by short-tandem repeat genotyping on December 3, 2012, and the method of culturing human umbilical venous endothelial cells (HUVEC; ref. 18). The methods to establish stably silenced clones of AMACR-amplified NMFH-1 and NMFH-2 cells are detailed in Supplementary Methods S2, using the pLKO.1-shAMACR (TRCN0000084113; TRCN0000084116) lentiviral vectors.

| **Table 1. Associations of AMACR expression and gene dosage with various clinicopathologic parameters in 105 patients with primary myxofibrosarcomas** |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| **AMACR expression** | **P** | **AMACR gene** | **P** |
| **Sex** | | | | |
| Male | 45 | 14 | 0.441 | 0.104 |
| Female | 32 | 14 | 0.449 | 0.240 |
| **Age, y** | | | | |
| <60 | 31 | 9 | 0.462 | 0.557 |
| ≥60 | 46 | 19 | 0.247 | 0.593 |
| **Location** | | | | |
| Extremity | 58 | 23 | 0.207 | 0.695 |
| Axial | 19 | 5 | 42 | 19 |
| **Tumor depth** | | | | |
| Superficial | 34 | 9 | 0.001a | <0.001a |
| Deep | 42 | 19 | 35 | 8 |
| **Surgical margin** | | | | |
| R0 | 49 | 14 | 49 | 14 |
| R1 and R2 | 28 | 14 | 34 | 8 |
| **FNCLCC grade** | | | | |
| Grade 1 | 41 | 4 | 42 | 3 |
| Grade 2 | 29 | 17 | 35 | 11 |
| Grade 3 | 7 | 7 | 6 | 8 |
| **AJCC stage** | | | | |
| Stage I | 21 | 2 | 20 | 3 |
| Stage II | 25 | 11 | 31 | 5 |
| Stage III | 27 | 12 | 27 | 12 |
| **Tumor size** | 6.24 ± 4.75 | 7.90 ± 5.50 | 0.058 | 0.086 |
| **Mitotic rate** | 8.95 ± 9.29 | 15.50 ± 13.93 | 0.022a | 0.041a |
| **AMACR exp.** | | | | |
| Low | 72 | 5 | 92 | 5 |
| High | 11 | 17 | 16 | 18 |

*aStatistically significant.

*bWilcoxon rank-sum test.
to silence AMACR expression and the pLKO.1-shLacZ (TRCN0000072223) as the control (Taiwan National RNAi Core Facility).

**Quantification of transcripts of AMACR and cell cycle–associated genes**

As detailed in Supplementary Method S3, real-time RT-PCR was performed using an ABI StepOnePlus System for cell line samples to measure AMACR mRNA abundance and to profile Cell Cycle RT-PCR Expression Array (PAHS-020; SABioscience) in search of potential downstream mediators of AMACR that promote cell-cycle progression. We used Western blotting to validate those genes differentially expressed in both NMFH-1 and NMFH-2 myxofibrosarcoma cell lines, with \( P < 0.0001 \) between AMACR-knockdown and control conditions and >1.75-fold changes.

The method for quantifying AMACR transcripts in LCM-isolated sarcoma cells from 16 fresh myxofibrosarcoma tissue samples has been described in our recent publication (6).

**Western blot analyses**

Western blotting assay was performed to evaluate the endogenous AMACR expression in three myxofibrosarcoma cell lines, the efficiency of AMACR knockdown in NMFH-1 and NMFH-2 cells, and the validation for potential downstream mediators of AMACR identified by RT-PCR Expression Array, as described in Supplementary Methods S4.

**Pharmacologic assays**

Ebselen oxide was obtained from Sigma. We seeded NMFH-1, NMFH-2, and CCD966SK fibroblasts in 96-well plates at a density of \( 5 \times 10^3 \) cells per well the day before treatment with vehicle control (0.9% saline) or ebselen oxide at indicated concentrations (10–160 \( \mu \)mol/L) for 72 hours.

**Functional assays**

To evaluate functional alterations associated with AMACR knockdown and treatment with ebselen oxide, we assessed various cancer phenotypes using bromodeoxyuridine (BrdUrd), cell-cycle kinetics, soft agar, annexin V/propidium iodine, wound healing, and HUVEC tube formation assays, as detailed in Supplementary Methods S5–S11.

**Animal xenografts**

This protocol was approved by the animal use committee (103101402). Detailed in Supplementary Method S12, NMFH-2 myxofibrosarcoma cells transfected with shAMACR versus shLacZ or treated with ebselen oxide (20 and 40 \( \mu \)mol/L) versus vehicle control were inoculated into the flanks of 8-week-old SCID mice \( (n = 8 \text{ for each condition}) \) to analyze the in vivo effects of AMACR expression and ebselen oxide, respectively. The tumor volume was calculated using the formula: \( V = \pi/6 \times \text{length} \times \text{width}^2 \text{ (mm}^2\text{)} \). Whole sections from formalin-fixed xenografted specimens were stained with anti-cyclin D1 (1:50; Epi-}

### Statistical analysis

We evaluated the associations of AMACR gene dosage and AMACR immunoexpression with each other and with clinicopathologic factors using the \( \chi^2 \) or Wilcoxon rank-sum test as appropriate. Follow-up data were available for 89 patients, with the median duration 30.6 months (range, 2–229). The endpoints were metastasis-free survival (MFS) and disease-specific survival (DSS). We compared univariate prognostic analyses using the log-rank test. In the multivariate Cox regression analysis, significant prognosticators with univariate \( P < 0.05 \) were generally included, except AMACR, in which only protein overexpression was adopted on account of its better univariate predictive power and interdependent covariate relationship with amplification. The Student \( t \) test was used to analyze quantitative RT-PCR, functional, and pharmacologic assays for cell and xenograft samples.

### Results

**Increased DNA copies and mRNA expression of the AMACR gene were identified in genomic and transcriptomic datasets of myxofibrosarcomas**

In prior genomic studies, copy number gain/amplification of 5p has been frequently found in myxofibrosarcomas (8, 19). Recently, we characterized the SKP2 oncogene on 5p13.2 for its clinical and biologic relevance (6). Residing in the same long arm region (5p15.1-p13.2) together with SKP2, AMACR on 5p13.3 (Fig. 1A) was also shown to display increased DNA copies in 40% of myxofibrosarcoma tissue and cell samples in our prior genomic profiling (6). In the public transcriptomic dataset of 34 myxofibrosarcoma versus nine nontumoral soft tissue samples, the log2 ratio of AMACR mRNA expression was differentially upregulated in tumor tissues in all three AMACR-specific probe sets (Fig. 1B; ref. 8). Relative to adjacent nontumoral soft tissues, the increase of AMACR transcript was reproducible in LCM-enriched myxofibrosarcoma cells by real-time PCR (6). The AMACR mRNA expression was significantly higher in the American Joint Committee on Cancer (AJCC) stage III cases \( (P = 0.039) \) and of marginal significance in the conjoint group of FNCLCC grade 2 and grade 3 cases \( (P = 0.061; \text{Fig. 1C}) \).

**AMACR gene amplification and protein overexpression were associated with each other, with unfavorable clinicopathologic factors, and worse outcomes**

Next, we analyzed the clinical relevance of the AMACR gene copy number and its protein expression in a validation set of independent primary myxofibrosarcomas, yielding 105 informative cases including 45 grade 1, 46 grade 2, and 14 grade 3 tumors (Fig. 2A and Supplementary Table S1). In the FISH assay, AMACR amplification \( (n = 22; 21\%) \) was strongly related to AMACR immunohistochemical...
overexpression ($n = 28; 26.7\%$; Fig. 2A; Table 1; $P < 0.001$). However, 39.2\% (11 of 28) of AMACR-overexpressing tumors were not amplified at the AMACR locus, implying alternative mechanism(s) upregulating AMACR overexpression. Furthermore, both the myxofibrosarcomas harboring an amplified AMACR gene and those overexpressing AMACR protein displayed significantly higher mitotic rates and histologic grades (Table 1). Univariately (Supplementary Table S2), worse MFS ($P = 0.0002$) in portending poorer univariate DSS (Fig. 2C) was inferior to that of AMACR overexpression ($P = 0.0002$). In multivariate comparison (Supplementary Table S2), AMACR overexpression remained independently prognostic of both MFS ($P = 0.001$) and DSS ($P = 0.007$). In a smaller cohort of 60 cases for comparison with previously published SKP2 (6), AMACR only showed a trend toward association with SKP2 in the immunoeexpression level (Supplementary Table S3), while its overexpression represented as an independent adverse prognosticator for both endpoints (Supplementary Table S4).
AMACR expression promoted myxofibrosarcoma cell growth and cell-cycle progression by modulating cyclin D1 and cyclin T2

To gain insight into the biology, we next characterized OH931, NMFH-1, and NMFH-2 myxofibrosarcoma cell lines for their endogenous AMACR expression. Using CCD966SK fibroblasts as the baseline reference, we found that endogenous expression levels of AMACR mRNA and protein were higher in myxofibrosarcoma cell lines, especially in NMFH-1 and NMFH-2 cells (Fig. 3A, left), consistent with the increased gene copies shown in our previous genomic profiling (GSE35483; ref. 6). RNA interference was used to decipher the functional effects of AMACR overexpression, and significant silencing of AMACR expression was achieved in selected stable clones of NMFH-1 (Fig. 3A, middle) and NMFH-2 cells (Fig. 3A, right).

Compared with shLacZ controls, the BrdUrd incorporation rates in both stable AMACR-silenced NMFH-1 and NMFH-2 cells were significantly reduced (Fig. 3B). These cells also displayed fewer and smaller soft-agar colonies (Fig. 3C). These findings together pointed to the growth-promoting role of AMACR, prompting us to explore its potential mediators on cell-cycle regulation. Despite no apparently upregulated genes in the RT-PCR profiling, mRNA expression of three cyclin genes, that is, CCND1, CCNT2, and CCNF, was significantly and consistently downregulated in both AMACR-knockdown myxofibrosarcoma cell lines (Supplementary Table S5). Western blotting assays validated the concomitant downregulation of cyclin D1 and cyclin T2, but not cyclin F, at the protein level (Fig. 3D). The finding of downregulated cyclin D1 suggests that cell-cycle progression may be arrested in the G1-phase in AMACR-knockdown myxofibrosarcoma cell lines. However, this inference was only substantiated by flow cytometric analysis in NMFH-1 cells, while a modest increase in the percentage of tumor cells in the G2–M phase was observed in NMFH-2 cells (Supplementary Fig. S1). Various functional assays were performed to assess whether AMACR expression contributes to other malignant phenotypes in addition to enhancing tumor proliferation. Compared with shLacZ controls, no apparent alteration in evasion of apoptosis (Supplementary Fig. S2), cell migration/invasion (Supplementary Fig. S3), and tumor angiogenesis (Supplementary Fig. S4) were observed in AMACR-knockdown NMFH-1 and NMFH-2 cells.

AMACR expression promoted growth of NMFH-2 xenografts

We further examined whether the growth-promoting effect of AMACR expression in vitro would also hold true in vivo, and compared the tumor growth kinetics in NMFH-2 xenografts with or without AMACR knockdown. The AMACR-knockdown group displayed a significantly smaller average tumor volume from day 2 onward, and this trend was magnified until sacrifice on day 36 (Fig. 4A). The excised xenograft tumor specimens were measurably lighter in

Figure 2. A, gradually increased cellularity, nuclear pleomorphism, and rates of mitosis are observed in representative grade 1 (left), grade 2 (middle), and grade 3 (right) myxofibrosarcomas (top row). No amplification, low-level amplification, and high-level amplification are detected by FISH assay targeting the AMACR gene, with the ratio of the red signal to the green signal being 1, 4, and >10 in the corresponding cases, respectively (middle row). Granular cytoplasmic immunoexpression of AMACR is consistent with the subcellular distribution of mitochondria and peroxisomes, and is classified as absent, increased, and overexpressed in representative tumors from grade 1 to grade 3 (bottom row). B and C, in log-rank univariate analyses, worse MFS (B) is strongly associated with AMACR amplification (left) and AMACR overexpression (right). Both factors are also significantly predictive of worse DSS (C), while AMACR overexpression (right) shows better predictive power.
weight, compared with the controls (Fig. 4B). Histologically (Fig. 4C), the control xenografts displayed spindle to pleomorphic cells in a fibromyxoid matrix, imparting a high-grade myxofibrosarcoma. In contrast, AMACR-knockdown NMFH-2 xenografts showed reduced cellularity, with fewer pleomorphic cells in a more fibrous matrix, and significantly reduced immunohistochemical expression of AMACR, cyclin D1, cyclin T2, and Ki-67 (all \( P < 0.001 \), Fig. 4C). In clinical samples, the significant associations of AMACR with these three markers were also confirmed (Supplementary Table S6 and Supplementary Fig. S5).

Ebselen oxide inhibited AMACR-expressing myxofibrosarcoma cells and xenografts via proteasome-mediated degradation of AMACR and induction of apoptosis

Ebselen oxide is a nonsubstrate-based covalent inactivator of AMACR, which has been shown to be selectively toxic to AMACR-expressing prostatic cancer cells in vitro but still lacks robust elucidation of mechanisms and in vivo assessment (20). To test whether AMACR is a supressible target in myxofibrosarcomas, we characterized the therapeutic effect of ebselen oxide on AMACR-expressing NMFH-1 and NMFH-2 cells, both of which, incubated for 72 hours at the indicated doses, displayed attenuated cell viability and selective susceptibility with IC_{50} between 15 and 30 \( \mu \)mol/L, in contrast to the relative resistance of fibroblasts (Fig. 5A). Ebselen oxide at 20 or 40 \( \mu \)mol/L for 24 to 72 hours did not significantly decrease AMACR mRNA levels (Fig. 5A) but it time-dependently attenuated AMACR protein expression in both myxofibrosarcoma cell lines, especially prominently in NMFH-1 cells with higher endogenous AMACR expression (Fig. 5B). On the basis of this, we used cyclohexamide, an inhibitor of nascent protein biosynthesis in eukaryotes, to clarify whether AMACR protein was downregulated posttranscriptionally by ebselen oxide. Unlike the nearly constant AMACR expression in the PBS-treated controls, ebselen oxide-induced NMFH-1 and NMFH-2 cells promptly showed a decline in AMACR protein levels (Fig. 5A) but it time-dependently attenuated AMACR protein expression in both myxofibrosarcoma cell lines, especially prominently in NMFH-1 cells with higher endogenous AMACR expression (Fig. 5B). On the basis of this, we used cyclohexamide, an inhibitor of nascent protein biosynthesis in eukaryotes, to clarify whether AMACR protein was downregulated posttranscriptionally by ebselen oxide. Moreover, treatment with ebselen oxide at 40 \( \mu \)mol/L induced...
significant suppression of cyclin D1, cyclin T2, and Ki-67 expression (Supplementary Fig. S6) and cell apoptosis in NMFH-1 and NMFH-2 cells with increased sub-G0–G1 cell populations (Fig. 5C and Supplementary Fig. S7). In vivo, the growth-inhibiting effect of ebselen oxide on xenografts was dose-dependent and became significant from 1 and 2 weeks posttreatment onward at 40 and 20 μmol/L, respectively (Fig. 5D, left). The PBS-treated xenografts displayed a high cellular proliferation of hyperchromatic pleomorphic cells with frequent mitotic activity. However, ebselen oxide–treated counterparts were characterized by reduced cellularity with necrotic changes, loss of AMACR expression, and increased TUNEL-positive \( (P \leq 0.001) \) apoptotic cells (Fig. 5D, right), with concomitantly reduced expression of cyclin D1, cyclin T2, and Ki-67 (Supplementary Fig. S6).

Discussion

On the basis of conventional comparative genomic hybridization and recent microarray-based genomic studies, 5p gain/amplification represents a nonrandom chromosomal alteration in several sarcoma types \( (6, 8, 19, 21) \), including myxofibrosarcomas \( (6, 8, 19) \). There are several long complex amplicons on 5p with multiple discontinuous cores encompassing potential driver genes \( (6, 8) \). Integration or cross-referencing between genomic and transcriptomic profiling datasets may simplify determination of genuine driver oncogenes, whose consistent overexpression can confer growth advantages on cancer cells in which they are amplified \( (8, 22–24) \). Among the candidate drivers on 5p, \textit{AMACR} was differentially upregulated in myxofibrosarcoma tissues, in both the published transcriptome and LCM-isolated sarcoma cells quantified for mRNA abundance \( (6, 8) \). In this series, we have characterized \textit{AMACR} as an amplified oncogene in myxofibrosarcomas that promotes tumor aggressiveness and disease progression through heightened cell proliferation. Moreover, we have substantiated the selective inhibition of ebselen oxide in \textit{AMACR}-overexpressing myxofibrosarcomas through the induction of proteasome-mediated degradation and cellular apoptosis \textit{in vivo} and \textit{in vitro}, hence supporting the therapeutic relevance of \textit{AMACR} in myxofibrosarcomas.

\textit{AMACR}, first identified to be aberrantly overexpressed in prostatic carcinomas, is indispensable in the catabolism of phytol-derived, branched-chain fatty acids \( (9–12, 25) \). To understand the growth advantage from the resultant metabolic deregulation, it is desirable to elucidate the molecular
basis underlying AMACR overexpression, because most malignancies increase the need for fatty acids as an energy source (9–12). Using FISH, we validated AMACR amplification in 21% of the myxofibrosarcomas, detecting this phenomenon for the first time in human cancers. This rate was lower than that of SKP2 amplification (38%) found by our previous study where we adopted quantitative DNA-PCR to analyze SKP2 gene dosage (6). Notably, AMACR amplification was reflected at the protein level and strongly correlated with immunohistochemical overexpression.
However, involvement of alterative regulatory mechanisms was likely in a subset of myxofibrosarcomas, because approximately 40% of AMACR-overexpressing myxofibrosarcomas lacked gene amplification. Actually, the expression level of AMACR in common carcinomas is mostly regulated by various transcriptional factors, such as C/EBP family members, Sp1, and ZNF202 (25–27). In the multivariate analysis of a subset cohort, AMACR overexpression, independent of SKP2, remained as the single adverse prognosticator, with an insignificant trend between two oncogenes in expression levels. This finding and validation of AMACR with a proliferation-promoting attribute reinforce AMACR as an oncogene indeed, not only a surrogate bystander coamplified with SKP2 in myxofibrosarcomas. However, we should not hastily underestimate the relevance of SKP2, given its reported pleiotropic oncogenic attributes (6) and the potential bias of fewer cases evaluated.

The biologic function of the amplification-driven AMACR overexpression remains undefined in mesenchymal neoplasms. Recently, several lines of evidence linked the racemase activity of AMACR to alterations in cancer cell behavior (28). RNA interference showed that high AMACR protein concentration promoted cell proliferation of prostate carcinomas through its enhanced activity, in an androgen-independent manner (28). In this series, both AMACR amplification and AMACR overexpression were associated with increasing histologic grades of myxofibrosarcomas, with AMACR overexpression notably predictive of worse prognosis, independent of grades and stages. These clinical implications are most probably ascribable to the proliferative attribute of AMACR in maintaining a malignant phenotype without apparent biologic effect on cell survival, migration, invasion, and angiogenesis. Specifically, shAMACR impaired BrdUrd uptake and anchorage-independent colony formation in vitro and inhibited the growth of derived NMFH-2 xenografts in vivo, demonstrating an autonomous growth-promoting role of AMACR in myxofibrosarcomas.

Given that sustained cell growth is a fundamental hallmark of cancer (29), it was interesting to identify cyclin D1 and cyclin T2 as potential mediators of AMACR, driving uncontrolled cell proliferation. As a prototypic D-type cyclin, cyclin D1 binds to cyclin-dependent kinases (CDK) 4/6 to phosphorylate retinoblastoma (Rb), thereby unleashing E2F family members to transactivate genes required for progression from the G1 to S-phase (30). By amplification or translocation, the constitutive overexpression of D-type cyclins or associated CDK4/6 is an established oncogenic aberration in various cancer types (7, 30–33), including myxofibrosarcomas with CDK6 amplification. In this study, AMACR overexpression in myxofibrosarcomas could increase cyclin D1 expression at the mRNA and protein levels, whereas the mechanisms underlying this regulatory link remain to be elucidated. Several lines of circumstantial evidence suggest that complex cross-talks between deregulated lipid metabolism and cyclin D1 expression may involve the β-catenin–mediated Wnt signaling, a pathway known to enhance transcriptional activation of cyclin D1 (34, 35). In the mantle cell lymphomas characterized by the translocation-driven cyclin D1 overexpression, the blockade of overexpressed fatty acid synthase, an enzyme catalyzing de novo synthesis of long-chain fatty acids, not only downregulates cyclin D1 but also β-catenin (34). Notably, AMACR-overexpressing hepatocellular carcinomas are associated with mutated β-catenin, implying that AMACR is a potential target of β-catenin (35).

Belonging to the C-type cyclins, cyclin T2 binds to its kinase partner CDK9 to form the positive transcription elongation factor b (p-TEFb) that promotes transcription elongation of myriad genes by phosphorylating the carboxyl-terminal domain of RNA polymerase II (36–38). Although cyclin T2 expression is essential for distinct genes that are themselves essential for embryonic development (39), neither its level nor associated CDK9 kinase activity fluctuates during the cell cycle (36, 37). However, the potential role of cyclin T2 in regulating G1–S transition is probably linked to Rb protein (38, 40), for which miR-29a or miR-142-3p could downregulate cyclin T2 expression, in turn decreasing the phosphorylated Rb level (40). Intriguingly, in myxofibrosarcoma cell lines and xenografts stably silenced against AMACR, expression of cyclin D1 and cyclin T2 significantly decreased at the mRNA and protein levels, with concomitantly reduced cell proliferation. The AMACR-induced cell-cycle progression in myxofibrosarcomas might be operated by more diverse mechanisms, because the G1 arrest was only found in the AMACR-knockdown NMFH-1 cells, while a G2/M–arresting effect was observed in the NMFH-2 counterparts.

Ebselen oxide is a nonsubstrate-based covalent inactivator of AMACR enzyme recently identified by high-throughput screening of approximately 5,000 compounds with the goal of developing a novel therapy against androgen-independent prostatic cancers (20). With a lower Kᵢ value, ebselen oxide appears more potent than the preceding AMACR inhibitors and selectively kills AMACR-overexpressing prostatic cancer cells (20), which are likely addicted to oncometabolites derived from AMACR-dependent pathways (41). Compared with fibroblasts, the susceptibility to ebselen oxide of AMACR-overexpressing myxofibrosarcoma was validated in vitro and extended for the first time in vivo with a dose-dependent effect, indicating a therapeutic window for specific targeting. Besides its known inactivating enzyme activity, it is mechanically intriguing to find the induction of increased apoptotic cells and proteasome-mediated degradation of AMACR protein. A combination of these effects may account for the antitumor activity of ebselen oxide in myxofibrosarcomas; there is still room for improving the drug potency of ebselen oxide, given its modest IC₅₀ values of approximately 15 to 30 μmol/L (42).

In short, AMACR overexpression is associated with adverse prognosis and is more pervasive than gene amplification in primary myxofibrosarcomas. AMACR has been proven in vitro and in vivo to be an amplification-driven oncogene, with its proliferation-promoting function being the primary oncogenic attribute in myxofibrosarcomas. AMACR overexpression contributes to tumor aggressiveness.
and indicates adverse prognosis. Inducing proteasome-mediated AMACR degradation and apoptosis, etselen oxide demonstrates selective cytotoxicity in AMACR-expressing myxofibrosarcoma cell lines and dose-dependent inhibition of derived xenografts, signifying that AMACR is a potential therapeutic target in myxofibrosarcomas.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Conceptual design: C.-F. Li, H.-Y. Huang
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-F. Li, F.-M. Fang, S.-H. Li, Y.-H. Wang, H.-Y. Huang
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Acknowledgments
The authors thank Drs. Bridge and Ogose for providing myxofibrosarcoma cells and Chang Gung genomics core laboratory for technical assistance (CMRPG860251).

Grant Support
This work was sponsored by Taiwan National Science Council (NSC96-2320-B-182-008-MY3 to H.-Y. Huang, NSC99-2628-B-182-003-MY3 to H.-Y. Huang, NSC99-2320-B-384-001-MY2 to C.-F. Li), Ministry of Health and Welfare (DOH100-THP-009-3, MOHW103-TM-111-102001 to C.-F. Li and H.-J. Kung), and Chang Gung Hospital (CMRPG870753, CMRPG8A0321, and XM10AC0061 to H.-Y. Huang).

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Received May 9, 2014; revised September 19, 2014; accepted September 20, 2014; published OnlineFirst November 10, 2014.
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