The \( p53 \)-binding Protein MDM2 Gene Is Differentially Expressed in Human Breast Carcinoma

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Abstract

The human \( p53 \)-binding protein murine double minute 2 (MDM2) is believed to function as a negative regulator of \( p53 \). The MDM2 gene was cloned and sequenced only recently and was found to be amplified in a variety of sarcomas. Although mutations in the \( p53 \) gene have been shown to occur in human breast carcinoma (HBC), no information is available on MDM2 gene expression in HBC. In this study we report for the first time that the MDM2 gene is differentially expressed in HBC. Our results demonstrate a correlation between the estrogen receptor (ER) status and the MDM2 mRNA levels. In contrast to the ER-negative cell lines, all the ER-positive cell lines were found to express higher levels of MDM2 mRNA. ER-positive ZR-75 cells express 30-fold higher levels of MDM2 mRNA than does the ER-negative cell line Hs578T. Estrogen enhanced albeit modestly the MDM2 mRNA levels in ER-positive MCF-7 cells. Estrogen enhancement of MDM2 mRNA levels was also observed in ER-negative MDA-MB-231 cells transfected with functional ERs. Our data thus suggest that estrogen may play an important role in HBC growth stimulation by modulating the expression of MDM2, which in turn may inactivate the \( p53 \) function.

Introduction

The human \( p53 \)-binding protein MDM2\(^3\) gene was cloned and sequenced only recently (1). The MDM2 gene encodes an mRNA of approximately 5.5 kilobases and the MDM2 gene itself was found to be amplified in a variety of sarcomas (1, 2). Originally isolated from murine fibroblast cells, MDM2 has been implicated in tumorigenicity (3, 4). It is now believed that the product of the MDM2 gene binds with the \( p53 \) protein and functionally inactivates it (1, 5). A wealth of information now suggests that, among the genetic abnormalities identified in wide array of tumors, mutations in the \( p53 \) gene are the most frequent (6–8). It is also believed that \( p53 \) regulates gene transcription by binding to DNA in a sequence-specific manner and thus abnormalities in \( p53 \) can result in aberrant regulation of genes by \( p53 \) (7). The identification of MDM2 as a negative regulator of \( p53 \) would suggest another potential mechanism for \( p53 \) inactivation in tumors in which \( p53 \) appears to be genetically intact (1, 5). Human breast carcinoma has been shown to harbor \( p53 \) mutations (Ref. 9 and references therein). In addition, a number of established human breast cancer cell lines have been shown to possess \( p53 \) mutations (Ref. 9 and references therein). The MDM2 gene expression in human breast carcinoma has not been previously investigated. This study was undertaken to investigate the expression pattern of the human MDM2 gene in breast carcinoma cell lines.

Materials and Methods

Materials. Most of the materials used in this study have been described previously (10). E2 was obtained from Steraloids, Inc. (Wilton, NH). \(^{32}P\)-dCTP (300 Ci/mmol) was purchased from Amersham (Arlington Heights, IL).

Cell Lines and Cell Culture. The MCF-7, ZR-75, and MDA-MB-231 cell lines were generous gifts of Dr. Marc Lippman (Lombardi Cancer Center, Washington, D.C.). The T47D, Hs578T cell lines were purchased from American Type Culture Collection (Rockville, MD). The cell culture conditions and preparation of charcoal-dextran and sulfatase-treated serum were as described previously (10).

DNA Probes and Southern and Northern Blot Analyses. The human MDM2 cDNA probe (1) was kindly provided by Drs. K. Kinzler and B. Vogelstein (Johns Hopkins University, Baltimore, MD). The human \( p52 \) (12) cDNA probe was purchased from American Type Culture Collection (Rockville, MD). RNA extraction and Northern blot analysis were essentially as described (13). Southern blot analysis and genomic DNA extraction were performed according to standard procedures (14). Genomic DNA from HBC cell lines analyzed in this study and from normal human stomach mucosa was digested with EcoRI, and the blots were probed with human MDM2 cDNA probe.

Stable Transfections of ER-negative MDA-MB-231. ER-negative MDA-MB-231 cells plated at a density of \( 1 \times 10^6 \) cells/100-mm dish were cotransfected with 10 \( \mu \)g of expression vector pSG5-HEO (15) and 2 \( \mu \g of the dominant selection vector pSV2neo (16) utilizing the calcium phosphate method as described in Sambrook et al. (14). The expression vector pSG5-HEO (kindly provided by Professor P. Chambon, Strasbourg-Cedex, France) carries the human ER coding region under the control of the SV40 promoter (15). Control cells were transfected with the pSV2neo vector alone. G418-resistant colonies were selected in medium containing 800 \( \mu \)g/ml G418 and were clonally expanded into mass culture. The ER-positive colonies were identified by Western immunoblotting utilizing monoclonal antibodies to human ER (kindly provided by Dr. G. L. Greene, University of Chicago) and colonies expressing ER levels comparable to the levels detected in MCF-7 cells were selected for further analysis.

Results and Discussion

Fig. 1 demonstrates the expression of the MDM2 gene in four ER-positive and three ER-negative HBC cell lines. In addition to an approximately 5.5-kilobase MDM2 mRNA as reported previously (1, 2), we also detected the expression of multiple bands in all the cell lines tested. Oliner et al. (1) have isolated multiple cDNAs for MDM2; whether the multiple bands for MDM2 noted in this study represent alternatively spliced forms of MDM2 mRNA or closely related genes remains to be elucidated. Fig. 1, however, clearly suggests that there is an apparent correlation between the expression of the MDM2 gene and the ER status of these cell lines. The MDM2 gene is expressed at a higher level in ER-positive cell lines than in the ER-negative cell lines. Quantitation of the 5.5-kilobase band representing MDM2 on Northern blots revealed that the ER-positive ZR-75 cell line expresses

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\(^3\) The abbreviations used are: MDM2, murine double minute 2; HBC, human breast carcinoma; ER, estrogen receptor; E2, 17\( \beta \)-estradiol; cDNA, complementary DNA; t\(_{1/2}\), half life.
the highest levels of MDM2 mRNA among all the cell lines examined; there was an approximate 30-fold difference in the expression levels of MDM2 mRNA noted in ZR-75 and the ER-negative Hs578T cells. Hs578T cells expressed the lowest levels of MDM2 mRNA among all the cell lines tested. Despite the fact that the ER-positive T47D cells had the lowest MDM2 mRNA levels among the ER-positive cell lines, they exhibited an approximate 2-fold higher (n = 3) MDM2 mRNA levels than that noted in the ER-negative MDA-MB-231 cells. It is of note that T47D cells contain very low levels of ER and they also contain dominant negative mutant variants of ER (Ref. 17 and references therein). To further investigate whether the differential expression of MDM2 mRNA as noted on Northern analysis was the result of gene amplification and/or rearrangements, Southern blot analysis was performed. The results obtained (data not shown) indicated that the high constitutive levels of MDM2 mRNA noted in ER-positive cells was not the result of gene amplification as has been found in a number of sarcomas (1, 2). In addition, no gross alterations were observed in the MDM2 gene in these cell lines when compared to DNA obtained from normal human stomach mucosa (data not shown). These findings clearly indicate that the differential expression of MDM2 mRNA noted in HBC cells was the result of differential regulation of the MDM2 gene at the transcriptional and/or at posttranscriptional level. Since there was an apparent correlation between the ER status and the levels of MDM2 mRNA, we sought to investigate the regulation of MDM2 gene by estrogen. MCF-7 cells are ER positive and have been studied extensively. These cells are growth stimulated by estrogen (18) and the regulation of a number of genes by estrogen has been investigated in these cells (Ref. 19 and references therein). MCF-7 cells were treated with a physiological concentration (1 nM) of E2 for different times and the Northern blot analyses revealed only a minimal effect of E2 on MDM2 mRNA abundance (data not shown). Growth factor-mediated induction of growth related genes such as c-fos, c-jun, and c-myc has been demonstrated in several systems (20, 21). Serum modulation of p33 at the mRNA and protein level has also been shown (Ref. 22 and references therein). MDM2 is considered a negative regulator of p53 and thus should play an important role in cell proliferation. In addition, we also found that the t1/2 of MDM2 mRNA is very short (40-45 min) in MCF-7 cells (data not shown) and is typical of the t1/2 for the mRNAs encoded by immediate early genes. We, therefore, also investigated the effect of serum on the modulation of MDM2 gene expression. MCF-7 cells were grown arrested by serum starvation and then treated with 15% fetal bovine serum for different periods of time. The results obtained (data not shown) demonstrated that the quiescent cells constitutively expressed MDM2 mRNA and that serum stimulation of MCF-7 cells exhibited only a modest modulation of MDM2 gene expression.

We have recently transfected ER-negative MDA-MB-231 cells with the expression plasmid pSG5-HEO encoding human ERs and characterized the clones that express functional ERs.4 We investigated whether the stable transfection of functional ERs modulate the MDM2 gene expression in ER-transfected MDA-MB-231 cells. Fig. 2 demonstrates that E2 treatment of ER-transfected MDA-MB-231 cells (clone4) resulted in an approximately 3.2-fold increase (n = 2) in the steady-state MDM2 mRNA levels. As expected, neither the parental nontransfected MDA-MB-231 cells nor the pSV2neo transfected cells responded to E2 treatment. E2 modulation of the known estrogen-regulated p52 gene (12) expression was also investigated and as can be seen (Fig. 2), E2 increased the endogenous p52 mRNA levels only in the ER-transfected MDA-MB-231 cells (Fig. 2), thus confirming the functionality of the transfected ERs. E2 modulation of MDM2
mRNA was further investigated in another ER-transfected MDA-MB-231 clone (clone5). Clone5 exhibits characteristics similar to clone4 in terms of the ER levels, growth behavior, and cell morphology. Similar treatment of clone5 with E2 also resulted in an approximately 3-fold increase in the steady-state MDM2 mRNA levels (data not shown). This suggests that the E2 effect on MDM2 mRNA levels appears specific and was due to the presence of functional ERs in the MDA-MB-231 cells since E2 did not modulate MDM2 mRNA in the wild-type or pSV2neo transfected MDA-MB-231 cells (Fig. 2; data not shown).

This is the first report that has investigated the expression of the MDM2 gene in HBC. In this report we have demonstrated that the MDM2 gene is differentially expressed in HBC cells and there appears to be a correlation between the ER status and the levels of MDM2 mRNA. MDM2 mRNA levels were approximately 30-fold higher in ER-positive cells than the levels of MDM2 mRNA. MDM2 mRNA levels were approximately 30-fold higher in ER-positive cells than the levels of MDM2 mRNA. The mechanism by which estrogen modulate MDM2 mRNA accumulation remains to be determined. We found that the MDM2 mRNA is very labile with a t1/2 of 40–45 min which is in close agreement with recently reported 30-min t1/2 of MDM2 protein (23). The estrogen effect on MDM2 mRNA levels in MCF-7 cells was, however, modest and the biological significance of this increase in MDM2 mRNA must await further study. Estrogen is a strong stimulator of HBC growth (18). ER-positive HBC cells are thought to represent an earlier stage in tumor progression than the more aggressive ER-negative cells (24, 25). The hormone-independent ER-negative HBC cells are mostly undifferentiated and have a high proliferative index (25). The basis of their more aggressive phenotype is not clear but is thought to be due to higher constitutive levels of secreted growth factors and the up-regulation of oncoproteins (25, 26). It is thus tempting to speculate that at an early stage in breast tumour progression p53 may still be intact but is negatively regulated by higher levels of MDM2 which is under hormonal control. Because of selective pressure towards a more aggressive phenotype, the cells tend to lose ERs and during this transition, they may also lose control of MDM2 expression and accumulate mutations in p53 gene. Consistent with this hypothesis would be a recent report (9) that studied a number of established and primary HBC cell lines and found that the primary tumors exhibited a lower incidence of p53 mutations. It was thus concluded that the occurrence of p53 mutations in HBC is a late event and often affects only a subset of tumors (9). Furthermore, the ER-positive MCF-7 and ZR-75 cells which we found (in this study) to express higher levels of MDM2 mRNA appear to contain wild-type p53 (9). A recent report by Allred et al. (27) studying 700 patients actually found a significantly higher incidence of p53 mutations in ER-negative breast tumors. This may also further support the notion that the ER-positive cells that have a lower incidence of p53 mutations may indeed have higher expression levels of MDM2. The study conducted by Allred et al. (27) also found that a number of tumors showed normal p53 sequence yet they expressed higher levels of p53. Wild-type p53 is very labile protein and has a very short t1/2 (Ref. 27 and references therein). Mutant p53 in contrast tends to accumulate in tumors as a more stable form (Ref. 27 and references therein). It may thus be possible that in tumors which exhibit the wild-type p53 sequence, the protein itself is stabilized and inactivated after its association with MDM2. Further studies are obviously indicated to investigate in detail the status of MDM2 in HBC.

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References

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